FABRICATION AND CHARACTERISATION OF ELECTROCHEMICAL BIOSENSORS FOR THE DETERMINATION OF CHOLESTEROL

By

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A Thesis Presented for the Degree of Doctor of Philosophy

University of Western Sydney

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TABLE OF CONTENTS

Certificate of Candidate \hspace{1cm} i
Disposition of Thesis \hspace{1cm} ii
Abstract \hspace{1cm} xii
List of Publications \hspace{1cm} xvi
List of Symbols and Acronyms \hspace{1cm} xvii
List of Figures and Tables \hspace{1cm} xix
Acknowledgments \hspace{1cm} xxx

CHAPTER 1 \hspace{0.5cm} LITERATURE REVIEW

1.1 GENERAL INTRODUCTION \hspace{1cm} 1
1.1.1 Structure of Cholesterol \hspace{1cm} 2
1.1.2 Cholesterol in the Body \hspace{1cm} 2
1.1.3 Functions of Cholesterol in the Body \hspace{1cm} 3
1.1.4 The Adverse Effects of Excess Cholesterol \hspace{1cm} 4
1.1.5 Cholesterol and Cardiovascular Disease \hspace{1cm} 5

1.2 DETERMINATION OF CHOLESTEROL \hspace{1cm} 6
1.2.1 Previous Methods of Detection \hspace{1cm} 6
1.2.2 Cholesterol Determination with Biosensors \hspace{1cm} 8
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.3</td>
<td>BIOSENSORS</td>
<td></td>
</tr>
<tr>
<td>1.3.1</td>
<td>Features and Benefits of Biosensors</td>
<td>11</td>
</tr>
<tr>
<td>1.3.2</td>
<td>The Origin of Biosensors</td>
<td>16</td>
</tr>
<tr>
<td>1.3.3</td>
<td>The Current Biosensor Design</td>
<td>18</td>
</tr>
<tr>
<td>1.4</td>
<td>ELECTROCHEMICAL METHODS OF DETECTION</td>
<td>20</td>
</tr>
<tr>
<td>1.4.1</td>
<td>Amperometric Biosensors</td>
<td>20</td>
</tr>
<tr>
<td>1.4.2</td>
<td>Potentiometric Detectors</td>
<td>24</td>
</tr>
<tr>
<td>1.4.3</td>
<td>Voltametric, Conductometric and Other Detectors</td>
<td>25</td>
</tr>
<tr>
<td>1.5</td>
<td>IMMOBILISATION METHODS</td>
<td>27</td>
</tr>
<tr>
<td>1.5.1</td>
<td>Adsorption</td>
<td>29</td>
</tr>
<tr>
<td>1.5.2</td>
<td>Covalent Bonding or Covalent Coupling</td>
<td>33</td>
</tr>
<tr>
<td>1.5.3</td>
<td>Entrapment</td>
<td>35</td>
</tr>
<tr>
<td>1.5.4</td>
<td>Encapsulation</td>
<td>38</td>
</tr>
<tr>
<td>1.5.5</td>
<td>Cross-linking</td>
<td>39</td>
</tr>
<tr>
<td>1.6</td>
<td>MEMBRANES FOR ENZYME IMMOBILISATION</td>
<td>41</td>
</tr>
<tr>
<td>1.6.1</td>
<td>Conducting Polymer Films</td>
<td>42</td>
</tr>
<tr>
<td>1.6.2</td>
<td>Non-Conducting Polymer Films</td>
<td>43</td>
</tr>
<tr>
<td>1.6.3</td>
<td>Composite Polymer Films</td>
<td>43</td>
</tr>
<tr>
<td>1.6.4</td>
<td>Conductive Polypyrrole Films and Biosensors</td>
<td>44</td>
</tr>
<tr>
<td>1.7</td>
<td>FABRICATION OF POLYMER FILMS</td>
<td>45</td>
</tr>
</tbody>
</table>
1.7.1 Solvent Casting 45
1.7.2 Spin Coating 45
1.7.3 Adsorption of Polymer Film onto an Electrode 46
1.7.4 Electropolymerisation 46

1.8 AIMS AND OBJECTIVES 48

CHAPTER 2 DEVELOPMENT OF A POLYPYRROLE-BASED CHOLESTEROL OXIDASE BIOSENSOR FOR CHOLESTEROL

2.1 INTRODUCTION 50

2.2 EXPERIMENTAL 52
2.2.1 Laboratory Conditions 52
2.2.2 Instrumentation 52
2.2.3 Glassware 53
2.2.4 Chemicals and Standard Solutions 54
2.2.5 Procedures 55
   (a) Electrode Preparation 55
   (b) Electropolymerisation of PPy-COD-[Fe(CN)6]3+ Film 55
   (c) Cholesterol Standard 56
   (d) Amperometric and Potentiometric Measurement 56
   (e) Electrode Storage 57

2.3 RESULTS AND DISCUSSION 57
2.3.1 Characterisation by Chronopotentiometry and Cyclic Voltammetry 57
(a) Chronopotentiometry 57
(b) Cyclic Voltammetry 59
2.3.2 Effect of Applied Potential ($E_{\text{app}}$) 61
2.3.3 Effect of Polymerisation Time and Current Density 63
2.3.4 Effect of Pyrrole Concentration and Cholesterol Oxidase Concentration 66
2.3.5 Effect of $[\text{K}_4\text{Fe(CN)}_6]$ Concentration 70
2.3.6 Comparison of Amperometric and Potentiometric Detection 70
(a) Amperometric Detection 72
(b) Potentiometric Detection 75

2.4 CONCLUSION 79

2.5 SUMMARY OF RESULTS 80

CHAPTER 3 FABRICATION OF A BOVINE SERUM ALBUMIN–GLUTARALDEHYDE-CHOLESTEROL OXIDASE BIOSENSOR FOR THE MEASUREMENT OF FREE CHOLESTEROL

3.1 GENERAL INTRODUCTION 81

3.2 EXPERIMENTAL 83
3.2.1 Chemicals and Standard Solutions 83
3.2.2 Procedures 84
   (a) Enzyme Immobilisation 85
   (b) Potentiometric Measurement 86
   (c) Optimisation of [COD], [BSA] and [GLA] 86
   (d) Film Drying 87
   (e) Storage Study 87
   (f) Pre-Treatment and Analysis of Serum Samples 87

3.3 RESULTS AND DISCUSSION 88
3.3.1 Optimisation of Components in the Sensing Layer 88
   (a) Optimisation of COD Concentration 89
   (b) Optimisation of BSA Concentration 92
   (c) Optimisation of GLA Concentration 95
3.3.2 Optimisation of Drying Time 98
   (a) Air-Drying Time 98
   (b) Accelerated Drying Time 100
3.3.3 Determination of Optimum Storage Conditions 102
   (a) Wet Storage 102
   (b) Dry Storage 103
3.3.4 Interference Study 103
   (a) Effect of Ascorbic Acid 105
   (b) Effect of Uric Acid 108
3.3.5 Linear Concentration Range and Minimum Detectable Amount 111
3.3.6 Percentage Recovery and Application to Serum Samples

3.4 CONCLUSION

3.5 SUMMARY OF RESULTS

CHAPTER 4 FABRICATION OF A CHOLESTEROL OXIDASE / CHOLESTEROL ESTERASE BIOSENSOR FOR THE MEASUREMENT OF TOTAL CHOLESTEROL

4.1 INTRODUCTION

4.2 EXPERIMENTAL

4.2.1 Chemicals and Standard Solutions

4.2.2 Procedures

(a) Procedures for Enzyme Immobilisation

(i) Incorporation of CE into BSA-GLA

(ii) Co-Immobilisation of COD and CE into BSA-GLA

Single-Layer Film

(iii) Co-Immobilisation of COD and CE into BSA-GLA

Bi-Layer Film

4.3 RESULTS AND DISCUSSION

4.3.1 Incorporation of CE into BSA-GLA Film – Optimisation of CE Concentration

viii
4.3.2 Effect of Phosphate Buffer Concentration

4.3.3 Co-Immobilisation of COD and CE into BSA-GLA for

Determination of Total Cholesterol in a Single-Layer Film

(a) Optimisation of COD Concentration

(b) Optimisation of CE Concentration

4.3.4 Co-Immobilisation of COD and CE of into BSA/GLA for

Determination of Total Cholesterol – Bi-Layer Film

4.3.5 Comparison of Single-Film and Bi-Layer Configurations

4.3.6 Interference Study

(a) Effect of Ascorbic Acid

(b) Effect of Uric Acid

4.3.7 Linear Concentration Range and Minimum Detectable Amount

4.3.8 Percentage Recovery and Application to Serum Samples

4.4 CONCLUSION

4.5 SUMMARY OF RESULTS

CHAPTER 5 DEVELOPMENT OF A HYBRID POLYPYRROLE BASED CROSS-LINKED/CHOLESTEROL OXIDASE BI-LAYER BIOSENSOR FOR THE MEASUREMENT OF FREE CHOLESTEROL

5.1 INTRODUCTION
5.2 EXPERIMENTAL

5.2.1 Procedures for Electrode Preparation

(a) Preparation of Bi-Layer Film

(i) Layer 1 – Polypyrrole – Chloride (PPy-Cl) film

(ii) Layer 1 – Polypyrrole – Nitrate (PPy-NO3) film

(iii) Layer 1 – Overoxidised PPy-NO3 film

(iv) Layer 2 – Incorporation of COD into BSA-GLA

5.3 RESULTS AND DISCUSSION

5.3.1 Characterisation of Polypyrrole Films by Chronopotentiometry and Cyclic Voltammetry

(a) Chronopotentiometry

(b) Cyclic Voltammetry

5.3.2 Dependence of Sensitivity on Type of Pyrrole Film in Layer 1

5.3.3 Dependence of Sensitivity on Layer 2 Mixture Volume

5.3.4 Interference Study

5.3.5 Linear Concentration Range and Minimum Detectable Amount

5.3.6 Percentage Recovery and Application to Serum Samples

5.4 CONCLUSION

5.5 SUMMARY OF RESULTS
ABSTRACT
ABSTRACT

During the course of this study, an extensive investigation was conducted into the measurement of free and total cholesterol by fabrication of cholesterol biosensors. Specific areas investigated in-depth included the immobilisation of enzymes into conducting polypyrrole (PPy) film, bovine serum albumin-glutaraldehyde (BSA-GLA) gel and a hybrid bi-layer of PPy and BSA-GLA. Key parameters for the reliable measurement of cholesterol were optimised. The optimum parameters were chosen based on sensitivity, resolution and reproducibility of the responses obtained for cholesterol.

Chapter 1 is an indepth literature review on cholesterol, methods of cholesterol determination, biosensors and the use of biosensors for cholesterol determination. Specific areas discussed in this chapter include the origin of cholesterol, the role of cholesterol in the body, as well as the link between elevated cholesterol levels and disease states in the body. The advantages and disadvantages of previous and current chemical methods of cholesterol determination are reviewed. The use of enzymatic methods of cholesterol determination specifically in the area of cholesterol biosensors as well as justification for further investigation in this area is also discussed.

In Chapter 2, a biosensor is developed by entrapment of cholesterol oxidase (COD) and a mediator, potassium ferrocyanide [K₄Fe(CN)₆], into a
PPy film during galvanostatic film formation. The optimum conditions for formation of the PPy-COD-Fe(CN)_6^-_ film include 0.3 M pyrrole, 25 units/mL COD, 5 mM K_4[Fe(CN)_6], a polymerisation time of 100 s and an applied current density of 0.5 mA/cm^2. Cholesterol oxidase was successfully incorporated into the polypyrrole film, on a platinum electrode. This was verified by chronopotentiometry and cyclic voltammetry. Two modes of detection, amperometric and potentiometric were investigated. The optimum applied potential for the amperometric biosensing of cholesterol was -200 mV vs Ag/AgCl (3 M KCl) in 0.05 M phosphate buffer. A comparison of the sensitivities for amperometric (2 x 10^{-4} mA/cm^2) and potentiometric detection (6.6 x 10^{-2} mV/μM) revealed that potentiometric detection was more sensitive and enabled detection of a wider concentration range of cholesterol. A minimum detectable amount 12.4 μM cholesterol and a linear concentration range between 12.4 and 247.5 μM were achieved with potentiometric detection. In comparison, amperometric detection gave a minimum detectable amount of 49.5 μM and a linear concentration range between 49.5 and 198 μM cholesterol.

Chapter 3 describes an alternate method for immobilisation of COD to improve the sensitivity of the cholesterol biosensor. This was accomplished by immobilisation of COD based on chemical cross-linking with GLA and BSA. Reliable potentiometric measurement of cholesterol was accomplished with a GLA concentration of 4.5% v/v, BSA concentration of 6.8% w/v, COD concentration of 23 units/mL and a film drying time of 30 minutes. The minimum detectable amount for this biosensor was ~2.5 μM which was almost five times lower than that
obtained with the PPy-COD-Fe(CN)$_6^{4-}$ biosensor. The achievable was between 2.5 and 25 µM. This biosensor was successfully used for the determination of cholesterol in blood serum.

Chapter 4 investigates the development of a cholesterol biosensor for total cholesterol measurement. For this purpose cholesterol oxidase and cholesterol esterase (CE) were co-immobilised in a BSA-GLA matrix. As outlined in Chapter 3 key parameters were optimised, for the reliable measurement of cholesterol. The optimum parameters for reliable potentiometric measurement of total cholesterol were 4.5% v/v GLA, 6.8% w/v BSA, 23 units/mL COD and 46 units/mL CE. Both single and bi-layer configurations were considered for fabrication of the biosensor. The bi-layer configuration was more sensitive. It also had an added advantage of allowing the immobilisation of the cholesterol esterase layer as the outer layer, which enables the catalysis of the first hydrolysis step in total cholesterol measurement. The bi-layer BSA-GLA-COD/BSA-GLA-CE biosensor also achieved a minimum detectable amount of 2.5 µM. The achievable linear concentration of cholesterol in the more sensitive range was between 2.5 and 32 µM cholesterol. This biosensor was also successfully used for the determination of cholesterol in blood serum.

In Chapter 5 a hybrid bi-layer electrode is described, with a polypyrrole inner layer and an outer layer of cholesterol oxidase chemically cross-linked with GLA and BSA. The most sensitive responses are obtained when the inner layer was polymerised with a mixture of 0.1 M KNO$_3$ and 0.1 M pyrrole solution. A 2 µL volume of the mixture for layer 2, which
composed of 4.5% v/v GLA, 6.8% w/v BSA and 23 units/mL COD, was spread onto the electrode to give the optimum film thickness. At this value an optimum drying time of ~15 minutes and a response equilibration time of ~24 minutes was obtained. Achievable minimum detectable amount of cholesterol with the PPy-NO₃/BSA-GLA-COD biosensor is 2.5 μM and a linear concentration range between 2.5 and 25 μM was achieved in the most sensitive range. This biosensor was also successfully used for the determination of cholesterol in blood serum.
LIST OF PUBLICATIONS

CONFERENCES PRESENTATIONS


The Eleventh Royal Australian Chemical Institute Convention (11RACIC: 6-11 February 2000, Canberra, ACT).


JOURNALS


LIST OF SYMBOLS AND ACRONYMS
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BSA – Bovine Serum Albumin
CE – Cholesterol Esterase
COD – Cholesterol Oxidase
CV - cyclic voltammogram
GLA – Glutaraldehyde
GOD – Glucose Oxidase
FIA – Flow Injection Analysis
HDL – High Density Lipoprotein
ISFET – Ion Sensitive Field Effect Transistor
LDL APOB - Low Density Lipoprotein Apolipoprotein B
LDL – Low Density Lipoprotein
MD – Mean Deviation
n – (n = 3) Number of samples analysed
o-PPD – Poly(o-phenylenediamine)
PEG – Polyethylene Glycol
PPy – Polypyrrole
PVC – Polyvinyl(chloride)
SAW – Surface Acoustic Wave
Std. – Standard (cholesterol)
~ - approximately
u - μ (micro) in graphs. (Unable to use symbol in Microsoft Office: Excel.)
LIST OF FIGURES
AND TABLES
LIST OF FIGURES AND TABLES

CHAPTER 1

Figure 1.1: The molecular structure of cholesterol. (page 2)

Figure 1.2: The biosensor concept. (page 12)

Figure 1.3: A glucose biosensor based on the Clark oxygen electrode. (page 17)

Figure 1.4: Schematic of the operation of biosensors. (page 19)

Figure 1.5: Schematic of adsorption. (page 29)

Figure 1.6: Schematic of covalent binding. (page 35)

Figure 1.7: Schematic of entrapment. (page 38)

Figure 1.8: Schematic of encapsulation. (page 39)

Figure 1.9: Schematic of cross-linking. (page 41)

Table 1.1 a: Reported cholesterol biosensors for free cholesterol determination. (page 9)

Table 1.1 b: Reported cholesterol biosensors for free and total cholesterol determination. (page 10)
Table 1.2: Features and benefits of biosensors. (page 15)

Table 1.3: Transducers commonly used in biosensors. (page 21)

Table 1.4: Some analytes that can be detected with amperometric enzyme electrodes. (page 23)

Table 1.5: Fundamental considerations in selecting a support and method of immobilisation. (page 30)

CHAPTER 2

Figure 2.1: Characterisation of (a) PPy-NO₃ and (b) PPy-COD Films by chronopotentiometry. (page 59)

Figure 2.2: Characterisation of (a) PPy-NO₃ and (b) PPy-COD Films in 0.1 M NaNO₃ by cyclic voltammetry. (page 61)

Figure 2.3: Influence of applied potential on the sensitivity of cholesterol response obtained with PPy-COD-Fe(CN)₆⁴⁺ biosensor. (page 63)

Figure 2.4: Influence of polymerisation time on the sensitivity of cholesterol response obtained with PPy-COD-Fe(CN)₆⁴⁺ biosensor. (page 65)

Figure 2.5: Influence of current density on the sensitivity of cholesterol response obtained with PPy-COD-Fe(CN)₆⁴⁻ biosensor. (page 66)
**Figure 2.6**: Influence of pyrrole concentration on the sensitivity of cholesterol response obtained with PPy-COD-Fe(CN)$_6^{4+}$ biosensor. (page 68)

**Figure 2.7**: Influence of COD concentration on the sensitivity of cholesterol response obtained with PPy-COD-Fe(CN)$_6^{4+}$ biosensor. (page 70)

**Figure 2.8**: Influence of K$_4$Fe(CN)$_6$ concentration on the sensitivity of cholesterol response obtained with PPy-COD-Fe(CN)$_4^4$. (page 72)

**Figure 2.9**: Calibration curves obtained for the PPy-COD-Fe(CN)$_6^{4+}$ biosensor by amperometric detection. (page 74)

**Figure 2.10**: Typical amperometric responses of PPy-COD-Fe(CN)$_6^{4+}$ biosensor to cholesterol. (page 75)

**Figure 2.11**: Calibration curves obtained for the PPy-COD-Fe(CN)$_6^{4+}$ biosensor by potentiometric detection. (page 77)

**Figure 2.12**: Typical potentiometric responses of PPy-COD-Fe(CN)$_6^{4+}$ biosensor to cholesterol. (page 78)

**Table 2.1**: Comparison of amperometric and potentiometric detection on the sensitivity of cholesterol response obtained with PPy-COD-Fe(CN)$_6^{4+}$ biosensor. (page 79)

**Table 2.2**: Results and optimum conditions obtained using amperometric and potentiometric detection on the sensitivity of the cholesterol response obtained with PPy-COD-Fe(CN)$_6^{4+}$ biosensor. (page 81)
CHAPTER 3

**Figure 3.1:** Influence of varying [COD] on the sensitivity of cholesterol response obtained with the BSA-GLA-COD biosensor. (page 90)

**Figure 3.2:** Influence of varying [BSA] on the sensitivity of cholesterol response obtained with the BSA-GLA-COD biosensor. (page 93)

**Figure 3.3:** Influence of varying [GLA] on the sensitivity of cholesterol response obtained with the BSA-GLA-COD biosensor. (page 96)

**Figure 3.4:** Influence of varying air-dry time in BSA-GLA-COD film on the sensitivity of cholesterol response. (page 99)

**Figure 3.5:** Comparison of storage conditions on the sensitivity of cholesterol response obtained with BSA-GLA-COD biosensor. (page 104)

**Figure 3.6:** Influence of ascorbic acid on the sensitivity of cholesterol response obtained with BSA-GLA-COD biosensor. (page 106)

**Figure 3.7:** Influence of uric acid on the sensitivity of cholesterol response obtained with BSA-GLA-COD biosensor. (page 109)

**Figure 3.8 a & b:** Calibration curves for cholesterol obtained with the BSA-GLA-COD biosensor. (page 112, 113)

**Figure 3.9:** Typical potentiometric responses obtained for the quantification of serum in cholesterol with the BSA-GLA-COD biosensor. (page 116)
Table 3.1: Composition of each layer for BSA-GLA-COD electrode. (page 85)

Table 3.2: Influence of varying [COD] on the sensitivity of cholesterol response obtained with the BSA-GLA-COD biosensor. (page 91)

Table 3.3: Influence of varying [BSA] on the sensitivity of cholesterol response obtained with the BSA-GLA-COD biosensor. (page 94)

Table 3.4: Influence of varying [GLA] on the sensitivity of cholesterol response obtained with the BSA-GLA-COD biosensor. (page 97)

Table 3.5: Influence of varying air-dry time in BSA-GLA-COD film on the sensitivity of cholesterol response. (page 101)

Table 3.6: Influence of [ascorbic acid] on the sensitivity of cholesterol response obtained with BSA-GLA-COD biosensor. (page 107)

Table 3.7: Influence of [uric acid] on the sensitivity of cholesterol response obtained with BSA-GLA-COD biosensor. (page 110)

Table 3.8: Sensitivity of BSA-GLA-COD biosensor in percentage recovery of free of cholesterol. (page 115)

Table 3.9: Determination of cholesterol in serum samples with the BSA-GLA-COD biosensor. (page 117)

Table 3.10: Summary of results and optimum conditions obtained using the BSA-GLA-COD biosensor for free cholesterol measurement. (page 119)
CHAPTER 4

**Figure 4.1:** Influence of varying [CE] in BSA-GLA-CE film on the sensitivity of cholesterol response. (page 128)

**Figure 4.2:** Influence of varying [phosphate buffer] on the sensitivity of cholesterol response obtained with BSA-GLA-CE electrode. (page 131)

**Figure 4.3:** Influence of varying [phosphate buffer] on the equilibration time of the cholesterol response obtained with BSA-GLA-CE electrode. (page 132)

**Figure 4.4:** Influence of varying [COD] in BSA-GLA-COD-CE single layer film on the sensitivity of the response for total cholesterol. (page 135)

**Figure 4.5:** Influence of varying [CE] in BSA-GLA-COD-CE single layer film on the sensitivity of the response for total cholesterol. (page 138)

**Figure 4.6:** Influence of single and bi-layer film configurations on the sensitivity of cholesterol responses of BSA-GLA-COD-CE and BSA-GLA-COD/BSA-GLA-CE biosensors. (page 142)

**Figure 4.7:** Influence of ascorbic acid on the sensitivity of cholesterol response obtained with the bi-layer BSA-GLA-COD/BSA-GLA-CE biosensor. (page 145)
Figure 4.8: Influence of uric acid on the sensitivity of cholesterol response obtained with the bi-layer BSA-GLA-COD/BSA-GLA-CE biosensor. (page 148)

Figure 4.9 a & b: Calibration curves obtained for the bi-layer BSA-GLA-COD/BSA-GLA-CE biosensor. (page 151, 152)

Figure 4.10: Typical potentiometric responses obtained for the quantification of serum in cholesterol with the bi-layer BSA-GLA-COD/BSA-GLA-CE biosensor. (page 155)

Table 4.1: Composition of CE layer. (page 124)

Table 4.2: Composition of sensing layer – single-layer film. (page 125)

Table 4.3: Compositions of sensing layers – bi-layer film. (page 126)

Table 4.4: Influence of varying [CE] in BSA-GLA-CE film on the sensitivity of cholesterol response. (page 129)

Table 4.5: Influence of varying [phosphate buffer] on the sensitivity of the cholesterol response obtained with BSA-GLA-CE electrode. (page 133)

Table 4.6: Influence of varying [COD] in BSA-GLA-COD-CE single-layer film the sensitivity of the response for total cholesterol. (page 136)
Table 4.7: Influence of varying [CE] in BSA-GLA-COD-CE single-layer film the sensitivity of the response for total cholesterol. (page 139)

Table 4.8: Optimised compositions of sensing layers for bi-layer film configuration. (page 140)

Table 4.9: Influence of single or bi-layer film configuration on the sensitivity of the combined enzyme biosensors for total cholesterol measurement. (page 143)

Table 4.10: Influence of [ascorbic acid] on the sensitivity of cholesterol response obtained with the bi-layer BSA-GLA-COD/BSA-GLA-CE biosensor. (page 146)

Table 4.11: Influence of [uric acid] on the sensitivity of cholesterol response obtained with the bi-layer BSA-GLA-COD/BSA-GLA-CE biosensor. (page 149)

Table 4.12: Sensitivity of bi-layer BSA-GLA-COD/BSA-GLA-CE biosensor in percentage recovery of total cholesterol. (page 154)

Table 4.13: Determination of cholesterol in serum samples with the bi-layer BSA-GLA-COD/BSA-GLA/CE biosensor. (page 156)

Table 4.14: Summary of results and optimum conditions obtained with the bi-layer BSA-GLA-COD/BSA-GLA/CE biosensor for total cholesterol measurement. (page 158)
CHAPTER 5

**Figure 5.1:** Characterisation of (a) PPy-NO$_3$ and (b) PPy-Cl films by
chronopotentiometry. (page 166)

**Figure 5.2:** Characterisation of (a) PPy-NO$_3$ and (b) overoxidised PPy-NO$_3$ films
by chronopotentiometry. (page 167)

**Figure 5.3:** Characterisation of (a) PPy-Cl and overoxidised PPy-NO$_3$ films by
cyclic voltammetry. (page 169)

**Figure 5.4:** Influence of type of pyrrole film in layer 1 on the sensitivity of bi layer
PPy-NO$_3$/BSA-GLA-COD biosensor for cholesterol measurement.
(page 171)

**Figure 5.5:** Influence of layer 2 mixture volume on the sensitivity of cholesterol
response obtained with bi-layer PPy-NO$_3$/BSA-GLA-COD biosensor.
(page 174)

**Figure 5.6:** Influence of layer 2 mixture volume on the drying time of bi-layer
PPy-NO$_3$/BSA-GLA-COD biosensor. (page 177)

**Figure 5.7:** Influence of layer 2 mixture volume on the equilibration time of
cholesterol response obtained with the bi-layer PPy-NO$_3$/BSA-GLA-
COD biosensor. (page 178)
**Figure 5.8:** Influence of ascorbic acid on the sensitivity of cholesterol response obtained with the bi-layer PPy-NO$_3$/BSA-GLA-COD biosensor. (page 180)

**Figure 5.9:** Influence of uric acid on the sensitivity of cholesterol response obtained with the bi-layer PPy-NO$_3$/BSA-GLA-COD biosensor. (page 183)

**Figure 5.10 a & b:** Calibration curves obtained for the bi-layer PPy-NO$_3$/BSA-GLA-COD biosensor. (page 186,188)

**Figure 5.11:** Typical potentiometric responses obtained for the quantification of serum in cholesterol with the bi-layer PPy-NO$_3$/BSA-GLA-COD biosensor. (page 190)

**Table 5.1:** Composition of layer 2 in PPy-NO$_3$/BSA-GLA-COD biosensor. (page 164)

**Table 5.2:** Influence of type of PPy-Film (Layer 1) on the performance of PPy-NO$_3$/BSA-GLA-COD bi-layer biosensor for free cholesterol measurement. (page 172)

**Table 5.3:** Influence of layer 2 mixture volume (BSA-GLA-COD) on the sensitivity of cholesterol response obtained with bi-layer PPy-NO$_3$/BSA-GLA-COD biosensor. (page 175)
Table 5.4: Influence of [ascorbic acid] on the sensitivity of cholesterol response obtained with the bi-layer PPy-NO₃/BSA-GLA-COD biosensor. (page 181)

Table 5.5: Influence of [uric acid] on the sensitivity of cholesterol response obtained with the bi-layer PPy-NO₃/BSA-GLA-COD biosensor. (page 184)

Table 5.6: Sensitivity of bi-layer PPy-NO₃/BSA-GLA-COD biosensor in percentage recovery study of free cholesterol. (page 189)

Table 5.7: Determination of cholesterol in serum samples with a bi-layer PPy-NO₃/BSA-GLA-COD Biosensor. (page 191)

Table 5.8: Summary of results and optimum conditions obtained using the bi-layer PPy-NO₃/BSA-GLA-COD biosensor for free cholesterol measurement. (page 194)
ACKNOWLEDGMENTS

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xxx
CHAPTER 1

LITERATURE REVIEW
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LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

Within the last two decades, an increasing number of publications have dealt with the determination of cholesterol in blood \[^1\] , serum and whole blood \[^2\] . This has stemmed from the discovery of the relationship between elevated cholesterol levels in the blood and disease states. Certain levels of cholesterol are essential for several functions in the body, but excessive amounts can contribute to several disease states such as arteriosclerosis, coronary heart disease, lipid metabolism dysfunction and hypertension \[^3\] - \[^12\] . Hence, the medical profession has shown increasing interest in monitoring blood and/or serum cholesterol levels in individuals who are genetically pre-disposed to such diseases.

Cholesterol, illustrated in Figure 1.1, is a large molecule with a complex nucleus and a waxy texture. There are two main sources of cholesterol. The body produces the majority of cholesterol according to its needs \[^8\] , however it may also absorbed from the consumption of foods like meat, seafood and extruded meat products. These foods contain cholesterol because of their animal sources \[^13\] . There is actually cholesterol present in all types of food that has any animal origin e.g. lard, whipped cream, milk, butter and cheese.
1.1.1 Structure of Cholesterol

![Molecular structure of cholesterol](image)

**Figure 1.1:** The molecular structure of cholesterol \(^{[14]}\).

1.1.2 Cholesterol in the Body

Cholesterol in the body is produced primarily in the liver, however small amounts are also produced in other organs of the body such as the adrenal capsules and the small intestine \(^{[6]}\). Some cholesterol is reused and reabsorbed in the liver where it is converted to bile acids, which help emulsify cholesterol. Cholesterol also travels to the small intestine where it helps digest fats.

Cholesterol is insoluble in blood so it combines with special proteins to form lipoproteins \(^{[7]}\). Only after being wrapped in these special protein-coats they are able to circulate in the blood stream. Cholesterol is
transported in blood from the liver to the peripheral tissues by low-density lipoproteins (LDL) \[^{12}\]. Any cholesterol that is not used by the peripheral tissues is gathered up by high-density lipoproteins (HDL) and brought back to the liver. The cholesterol is then reabsorbed by the liver and along with dietary cholesterol participates in digestion \[^{6,15}\].

**1.1.3 Functions of Cholesterol in the Body**

Cholesterol plays a role in digestion by contributing to the formation of bile acid, which allows fat emulsification and digestion \[^{7}\]. Cholesterol is also a component of plasma because it circulates in the blood in the form of HDL and LDL. It also has a role in the formation of body tissues and supplies some of the essential constituents of cell membranes \[^{10}\]. Furthermore, it is also essential in the proper functioning of the nervous system. It is found abundantly in nerve cells where along with other lipids it enters into the composition of the myelin sheath, which is the fatty tissue around nerve fibres \[^{6,10,16}\]. Hence, it is an active participant in the functioning of the brain. The brain and the spinal column are rich in cholesterol. Cholesterol is also active in the formation of sex hormones in men and women as well as in the production of adrenal cortical hormones \[^{7}\]. There is good evidence that cholesterol is essential to life as it carries out essential exchanges to maintain the body in working order \[^{6,7,15}\]. Under normal functions cholesterol may be used properly and any surplus eliminated. However, as mentioned previously the presence of excessive amounts of cholesterol can contribute to disease states in the body\[^{11,12,17}\].
1.1.4 The Adverse Effects of Excess Cholesterol

There may be a genetic factor associated with the presence of excessive levels of cholesterol in the body \[^{11}\]. Sometimes cholesterol is not processed properly resulting in high blood cholesterol levels (hypercholesterolaemia) \[^{6,11,15}\]. When the processing of cholesterol is interrupted it sets off a chain reaction of events, leading to a problem in the gall bladder. Cholesterol is contained in bile as well as in bile acid. When bile-cholesterol is in excess, relative to bile acid, in bile, the cholesterol precipitates and gallstones are formed \[^{9}\]. As the gallstones move through the canals they cause excruciating pain in the form of spasms. If the passage becomes obstructed and the bile cannot travel through the intestine, reflux of bile to the liver occurs. The resulting effect of this is jaundice \[^{6}\].

Hypercholesterolaemia and persistently high blood cholesterol levels may lead to cardio-vascular disease. In February 1997 a survey \[^{13}\] conducted by The National Heart Foundation reported that 47 percent of males and 39 percent of females in Australia between the ages of 20 and 65 years have high a blood cholesterol levels of $\geq 6.5 \text{ mM} \[^{13}\]$. The proportion of men and women with high blood cholesterol levels increases dramatically with age. Between the ages of 65 - 69 years, 62 percent of men and a shocking 97 percent of women have high blood cholesterol levels. The desirable blood cholesterol level has been set at less than 5.5 mM \[^{13}\]. The Foundation urges Australians to lower fat intake to thirty percent of each day’s energy intake.
1.1.5 Cholesterol and Cardiovascular Disease

The main consequence of persistently high blood cholesterol levels is atherosclerosis \[^{19}\]. It is a disease where the arteries harden over time. Cholesterol is deposited in fibrous plaques on walls of the arteries, thereby decreasing the wall diameter. This may lead to a block of the blood supply to the arteries. Plaques occur most commonly in arteries that supply the heart with blood \[^{8,17,18}\]. A blockage in this region would cause angina pectoris, which could lead to a heart attack. When the cerebral and or cervical arteries become blocked the result may be stroke, paralysis or infarction of the brain. Blockage of the lower limbs could lead to gangrene and possible amputation of the limb \[^{8,10}\].

Cholesterol from LDL tends to attach itself to arteries as it circulates from the liver to the peripheral tissues. There is a common misconception that LDL is bad cholesterol. LDL is not actually cholesterol, but simply a bad carrier of cholesterol in blood \[^{6}\]. The other carrier of cholesterol, HDL, sweeps out the excess from the arteries and delivers it back to the liver to be degraded.

The adverse effects of excess cholesterol as described above result from the interruption of the processing of cholesterol \[^{6,9,15,20}\]. This may be due to genetic reasons, where some families are pre-disposed to adverse effects from excess cholesterol. Alternatively it could be due to excess consumption of cholesterol rich foods \[^{13}\].
1.2 DETERMINATION OF CHOLESTEROL

1.2.1 Previous Methods of Detection

The determination of cholesterol is usually performed, by employing the enzymes cholesterol oxidase (COD) and cholesterol esterase (CE). The CE catalyses the hydrolysis of esterified cholesterol to free cholesterol and its fatty acid derivative as shown in equation 1.1, while the COD catalyses the oxidation of cholesterol as seen in equation 1.2. This is important for the determination of total cholesterol since approximately seventy percent of cholesterol in blood is esterified by fatty acids [3,21].

\[
\text{cholesterol fatty acid ester} \xrightarrow{\text{CE}} \text{cholesterol + fatty acid} \quad (1.1)
\]

\[
\text{cholesterol} \xrightarrow{\text{COD}} \text{4-cholest-3-en-1-one} + \text{H}_2\text{O}_2 \quad (1.2)
\]

In optical methods for cholesterol measurement, the reaction of the COD is coupled to another enzymatic reaction (with catalase or peroxidase) in which a dye is produced [3]. The change in absorbance or fluorescence measured can then be related to the cholesterol concentration [17,22-24]. The first optical biosensor for cholesterol was based on the detection of
oxygen consumption during the enzymatic reaction (as illustrated in equation 1.2) by using a dye whose fluorescence was quenched by molecular oxygen \[25\].

To date cholesterol has been determined almost exclusively by chemical methods \[21,26\] that are based on spectrophotometry \[27\] or colorimetric determination \[28\], which are not specific and require sample pre-treatment such as separation and lipid extraction. Chemical determination is not only time-consuming but may also lead to a wide range of error as well. The procedure is often complicated and drastic reagents are often used \[26,4\]. The majority of cholesterol assays involving colorimetric methods are often preceded by extraction into many organic solvent combinations and concentrated acids, such as sulfuric acid, acetic anhydride and acetic acid, occasionally involving digitonin precipitation \[28\].

Other methods available for cholesterol determination include ultracentrifugation \[19\], electrophoresis, HPLC and precipitation-based methods \[5,29\]. Currently, precipitation-based methods are routinely used for cholesterol determination in blood. HDL cholesterol is first separated by precipitating apoprotein B-containing lipoproteins, such as LDL and VLDL, from serum by using a combination of a polyanion and a divalent cation, such as dextran sulfate/manganese chloride or phosphotungstate/magnesium chloride \[5\]. The supernatant is then analysed for cholesterol using bichromatic measurement at 520 and 700 nm. The relevant equations for this reaction are 1.1 and 1.2 above and 1.3 below \[30\]. Such precipitation-based methods are, however, time consuming and are not amenable to automated analysis.
2 H₂O₂ + 4-aminophenazine + phenol \[\xrightarrow{\text{peroxidase}}\] 4-(p-benzoquinone-monoimino)-phenazine + 4 H₂O (1.3)

1.2.2 Cholesterol Determination with Biosensors

There is a great need for the development of a convenient and reliable method for measuring cholesterol concentrations in various samples without any pre-treatment. One such method capable of accomplishing this goal is based on the use of biosensors. These devices are usually simple and inexpensive to prepare and so far their use for analysis of a range of substances has shown that they require minimum sample pre-treatment \(^{[31]}\).

At present there is considerable interest in the development of cholesterol biosensors for the various reasons alluded to previously. Two types of cholesterol determination are possible: firstly free cholesterol determination requiring the immobilisation of COD; and secondly total cholesterol determination, which requires the immobilisation of both COD and CE. However, the accuracy of the technique is dependent on the sensitivity on the mode of detection and the selectivity of the immobilised COD-CE component. The versatility in the immobilisation methods and media available, for the immobilisation of COD or COD and CE, are illustrated in the examples listed in Table 1.1. Despite the successful development of these cholesterol biosensors with favourable detection limits and fast response times for the determination of cholesterol being reported, none are commercially available at present. Tables 1.1 (a) and 1.1 (b) show some of the cholesterol biosensors that have been reported to date.
**Table 1.1 (a):**

**Reported cholesterol biosensors for free cholesterol determination**

<table>
<thead>
<tr>
<th>Description of Electrode</th>
<th>Detection Mode</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>COD entrapped in overoxidised PPy/poly(o-phenylenediamine)</td>
<td>Amperometric</td>
<td>32</td>
</tr>
<tr>
<td>immobilisation of COD onto disposable reagentless screen-printed amperometric biosensor strip on a PVC support</td>
<td>Amperometric</td>
<td>4</td>
</tr>
<tr>
<td>electropolymerisation of PPy-COD with charge transfer mediators</td>
<td>Amperometric</td>
<td>33</td>
</tr>
<tr>
<td>entrapment of COD in PPy layer of multi-layer PPy/o-PPD configuration</td>
<td>Amperometric</td>
<td>34</td>
</tr>
<tr>
<td>covalent binding of COD to oxidase-peroxidase modified electrode</td>
<td>Amperometric</td>
<td>35</td>
</tr>
<tr>
<td>electrodeposition on catalytic Pd particles</td>
<td>Amperometric</td>
<td>36</td>
</tr>
<tr>
<td>Nafion modified PPy-COD</td>
<td>Amperometric</td>
<td>37</td>
</tr>
<tr>
<td>collagen immobilised COD</td>
<td>Amperometric</td>
<td>21, 38</td>
</tr>
<tr>
<td>electropolymerisation of PPY-COD</td>
<td>Amperometric</td>
<td>3</td>
</tr>
</tbody>
</table>
**Table 1.1 (b):**

Reported cholesterol biosensors for free and total cholesterol determination

<table>
<thead>
<tr>
<th>Description of Electrode</th>
<th>Detection Mode</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>COD-CE entrapped in overoxidised PPy film</td>
<td>Amperometric</td>
<td>39</td>
</tr>
<tr>
<td>coupling of CE-COD-Peroxidase and detection by tubular carbon electrodes</td>
<td>Amperometric</td>
<td>40</td>
</tr>
<tr>
<td>adsorption of COD on glass beads, CE in carrier solution of flow system</td>
<td>Amperometric</td>
<td>41</td>
</tr>
<tr>
<td>COD-CE pre-adsorption via Lapoinya clay additives</td>
<td>Amperometric</td>
<td>42</td>
</tr>
<tr>
<td>electropolymerisation in PPy matrix, immobilisation on modified carbon paste electrodes</td>
<td>Amperometric</td>
<td>43</td>
</tr>
<tr>
<td>COD-CE attachment to pre-activated nylon membrane</td>
<td>Fibre Optic</td>
<td>24</td>
</tr>
<tr>
<td>COD-CE cross linking using BSA-GLA on a gold electrode (FIA)</td>
<td>Amperometric</td>
<td>44</td>
</tr>
<tr>
<td>immobilisation of COD and CE on graphite - 70% teflon matrix</td>
<td>Amperometric</td>
<td>45</td>
</tr>
</tbody>
</table>
1.3 BIOSENSORS

The definition of a chemical sensor is a device that transforms chemical information, such as the concentration of specific analytes into an analytically useful electrical signal \([31,46,47]\). Biosensors are a sub-set of chemical sensors. A biosensor may be defined as a compact analytical device containing a biological or biologically derived sensing element (e.g. enzymes, antibodies, micro-organisms or DNA), either integrated with or in intimate contact with a physicochemical transducer (e.g. electrochemical, optical, thermometric or piezoelectric) \([48,49]\). The usual aim is to produce a continuous or semi-continuous digital electronic signal, which is proportional to a specific chemical or groups of chemicals. The devices may be configured to give qualitative or quantitative information. Figure 1.2 shows an analyte selective interface in close proximity/integrated with a transducer, whose function it is to relay the interaction between the surface and analyte either directly, or through a chemical mediator.

1.3.1 Features and Benefits of Biosensors

As the literature on biosensors continues to diversify \([50-53]\) there is an increasing interest for industry to critically evaluate and invest in focused areas of biosensors. Initially interest was stimulated by the needs in medical and clinical studies to determine compounds, such as glucose in whole blood, but there are now wider applications in bioprocess monitoring, food and environmental analysis \([54]\). Several biosensors for
**Figure 1.2:** The biosensor concept. Reproduced from [46].
the determination of various substances, such as cholesterol \([3, 4, 21, 32-45]\), urea \([55-58]\), glucose \([59-102]\), L and D-amino acids \([103]\), DNA \([48, 49]\) and sulfite \([104, 105]\) have been widely reported.

Pocket-sized devices capable of on the spot measurement of a wide range of analytes is very appealing to the food industry. Equally attractive is the possibility of on-line monitoring of complex biochemical parameters during production or processing of foods. The ability to provide continuous data on a specific analyte can be invaluable for optimisation and / or control. The relevance of biologically based sensing systems to the study of the effects of toxins, make biosensors ideal candidates for a broad range of alarms \([31, 106-108]\). On the other hand, the exquisite specificity of some enzymes, can distinguish between stereo isomers of the same compound to give precise information about the contents of samples \([48]\). Hence, specific quantification is also possible, even in the presence of chemically related compounds. In addition, the affinity of the receptor to the analyte, and in the case of a catalytic system, its chemical turnover, are so high that rather sensitive determination is possible. When combined with a suitable transducer, the biochemical reaction is transformed into an electrical signal, allowing its immediate use for documentation and control.

Increasing concern about chronic exposure to very low levels of residues necessitates the development of ultra-sensitive assays for the analytes, such as pesticides, herbicides, antibiotics and hormones. A key technology here is the construction of immunosensors, which may also
find application in the medical, food and pharmaceutical industries especially for speciation and microbial identification \cite{48}.

A summary of some of the features including: target specificity, electronic integration, fast response time, continuous measurement and selective measurement in complex sample matrices or samples as well as the benefits of biosensors are highlighted in Table 1.2 \cite{46,48,49}.

The distinct advantage of using enzymes as the biocomponents in sensor development is that greater analyte specificity is achieved. The high specificity reduces the need for pre-treatment of samples so that direct analysis may be carried out regardless of the sample matrix or complexity \cite{109}. Ideally enzymes with absolute specificity would be preferable for analytical use \cite{110}. Examples of these include cholesterol oxidase, glucose oxidase, sulphite oxidase, urease and urate oxidase. However, knowledge of the range of potential substrates of the reagent enzymes of interest is essential so that possible interferences in the assay could be identified and adjusted for accordingly \cite{110}. Nevertheless, the most important feature of enzymes is that they are effective catalysts for biological systems. They are valuable as tools in analytical chemistry and have also become an integral feature in biosensor technology.


Table 1.2:

Features and benefits of biosensors

<table>
<thead>
<tr>
<th>Feature</th>
<th>Benefit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Target Specificity</td>
<td>Versatility, sensitivity and selectivity (from definitive identification of single compound to broad range)</td>
</tr>
<tr>
<td>Electronic Integration</td>
<td>Elegant and compact instrument design resulting in ease of use with easier data processing and versatility</td>
</tr>
<tr>
<td>Selective Measurement in Complex Matrices/Samples</td>
<td>Simplicity, reproducibility, ease of use and decreased user time because no sample preparation needed</td>
</tr>
<tr>
<td>Fast Response time</td>
<td>Increased efficiency and decreased user time with automated analysis</td>
</tr>
<tr>
<td>Continuous Measurement</td>
<td>Increased accuracy and efficiency as well as improved precision</td>
</tr>
<tr>
<td>Compact Size</td>
<td>Portable, multiple simultaneous assays, inexpensive, ease of use and versatility</td>
</tr>
<tr>
<td>Mass Production</td>
<td>Inexpensive, disposable (hygienic), can be widely dispersed and increase revenues for manufacturer</td>
</tr>
<tr>
<td>Electrochemical Engineering</td>
<td>Reproducible</td>
</tr>
</tbody>
</table>

Adapted from references [46,48,49].
1.3.2 The Origin of Biosensors

This device was originally referred to as an enzyme electrode and was first described \(^{[11]}\) by Clark and Lyons in 1962 for the determination of glucose. In the initial work in this area oxygen was used as the oxidising agent. The consumption of oxygen was monitored at the platinum electrode. A voltage of -0.7V is applied between the platinum cathode and the silver anode, sufficient to reduce the oxygen, and the cell current which is proportional to the oxygen concentration, is measured. The decrease in the current measurement (oxygen concentration) is proportional to the glucose concentration. The oxygen electrode uses an oxygen permeable membrane, such as cellophane or polyethylene, which covers the electrode surface. A layer of GOD is then placed on top with a second membrane, such as cellulose acetate, as shown in Figure 1.3. The substrate, glucose solution and oxygen can penetrate the first membrane to react with the enzyme to form products. Only the remaining oxygen can penetrate the second membrane to be measured at the electrode \(^{[11]}\).

Early work on biosensor design utilised biologically active components that were physically entrapped in a membrane or in a polymer film. In some of these works, the biological components were fixed via direct adsorption or by covalent bonding \(^{[11]}\). The general construction of enzyme electrodes in the earlier studies was based on the Clark oxygen electrode \(^{[11]}\).
The glucose oxidation reaction, catalysed by glucose oxidase (GOD) is:

\[
\text{Glucose} + \text{O}_2 + \text{H}_2\text{O} \rightarrow \text{gluconic acid} + \text{H}_2\text{O} \quad (1.4)
\]

At the Cathode:

\[
\text{O}_2 + 2\text{e}^- + 2\text{H}^+ = \text{H}_2\text{O} \quad (1.5)
\]

**Figure 1.3:** A glucose biosensor based on the Clark oxygen electrode. Reproduced from\textsuperscript{112}. 

17
1.3.3 The Current Biosensor Design

A biosensor is composed of two main components. One is the transducer, which provides the analytical signal and the other is a biochemical reagent, which provides the selectivity \cite{46,47,54}. These two components are in intimate contact with one another to provide a stand-alone sensor that relates analyte concentration to a measurable signal \cite{113}. The biochemical reagent typically converts or helps to accelerate the conversion of the analyte of interest into another chemical species and/or physical property that is sensed and then transformed into an electrical signal by the transducer. In this case the transducer is an electrode. In an ideal situation, where the sample matrix is not too complex, this would be accomplished without pre-treatment or the addition of any reagents. The operation of a typical biosensor, is illustrated in Figure 1.4 \cite{114}. The biosensor lifetime, stability, reproducibility and calibration requirements are influenced significantly by the chosen biological component.

An enzyme based biosensor is composed of an immobilised enzyme at the surface of an electrochemical sensor. Basically the enzyme reacts with a substrate and consumes a co-reactant as illustrated below.

$$\text{Substrate} + \text{Co-Factor} \xrightarrow{\text{enzyme}} \text{Product(s)} \quad (1.6)$$

Either of the steps described above, may be monitored electrochemically.
Figure 1.4: Schematic of the operation of biosensors.
Reproduced from [114].
The transduction element of a biosensor must be capable of converting a specific biological reaction (binding or catalytic) into a response which can be processed into a useable signal. This element must also be suitable for the immobilisation of the biological component at or close to its surface. Table 1.3 outlines the most commonly used transducers [54].

1.4 ELECTROCHEMICAL METHODS OF DETECTION

Biosensors may be classified by the detection methods used to analyse the compound(s) of interest. Electrochemical techniques, such as amperometry, potentiometry and voltammetry are the most commonly used detection methods in biosensing. This is because of their selectivity, sensitivity and ability to detect low concentrations of analyte(s) without prior pre-treatment. An added advantage of these techniques is the low cost required for the equipment.

Other techniques such as coulometric, conductometric, pulsed amperometry and ac voltammetry have not been as widely investigated for biosensing applications. The specific features and advantages of the common electrochemical detection methods are described below.

1.4.1 Amperometric Biosensors

Amperometry was the basis of the first biosensor described by Clark and Lyons in 1962, in which GOD was immobilised next to a Clark oxygen electrode [111]. It has since continued to be the most popular approach to biosensing, largely due to its inherent simplicity, low cost,
## Table 1.3:
Transducers commonly used in biosensors

<table>
<thead>
<tr>
<th>Transducer</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Electrochemical</strong></td>
<td></td>
</tr>
<tr>
<td>Amperometric</td>
<td>Clark oxygen electrode, chemically modified electrodes</td>
</tr>
<tr>
<td>Potentiometric</td>
<td>Ion selective electrodes, field effect transistors</td>
</tr>
<tr>
<td>Conductimetric</td>
<td>Platinum electrodes</td>
</tr>
<tr>
<td><strong>Optical</strong></td>
<td>Optical fibre, evanescent fields devices</td>
</tr>
<tr>
<td><strong>Acoustic</strong></td>
<td>Piezoelectric crystals, surface acoustic wave devices</td>
</tr>
<tr>
<td><strong>Thermal</strong></td>
<td>Thermistor, thermopile</td>
</tr>
</tbody>
</table>

Adapted from [54].
ease of mass production and availability of instrumentation. The technique involves the measurement of resulting cathodic or anodic current with the application of a fixed potential to the biosensor. Detection is based on chemical species that are either reduced or oxidised at the applied potential \[31,48\]. The corresponding oxidising or reducing current produced is usually proportional to the concentration of the species of interest \[115\]. Table 1.4 shows a list of some of the substances that can be analysed by amperometric detection with enzyme electrodes.

The signal obtained is dependent on the rate of mass transfer to the electrode surface. Hence, it is common to use a diffusion barrier to minimise the variations due to turbulence and to extend the linear range of the sensor. In the simplest mode of operation, the consumption of a co-reactant or the release of an electroactive product due to a biocatalytic reaction can be monitored directly at an inert working electrode such as a platinum wire \[48\].

A popular theme at present is modification of the electrode material with electron donors or acceptors. The aim is to provide a charge transfer pathway between the biocatalytic component and the electrode surface. An added advantage of this approach is that it overcomes the oxygen dependence of certain enzymatic reactions, such as those based on oxidase acceptors \[48,106\]. In general, enzymes are very specific and the primary source of interference is derived from electroactive species, which may diffuse to the sensor surface and be oxidised.
Table 1.4:

Some analytes that can be detected with amperometric enzyme electrodes

<table>
<thead>
<tr>
<th>Analyte</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>ethanol</td>
<td>116</td>
</tr>
<tr>
<td>lactate</td>
<td>117</td>
</tr>
<tr>
<td>aspartate</td>
<td>118-119</td>
</tr>
<tr>
<td>allopurinol</td>
<td>120</td>
</tr>
<tr>
<td>catechol</td>
<td>121</td>
</tr>
<tr>
<td>nystatin</td>
<td>122</td>
</tr>
<tr>
<td>tricyclic antidepressants</td>
<td>123</td>
</tr>
<tr>
<td>pyruvate</td>
<td>124</td>
</tr>
<tr>
<td>3-hydroxybutyrate</td>
<td>125</td>
</tr>
<tr>
<td>choline</td>
<td>126</td>
</tr>
<tr>
<td>acetyl choline</td>
<td>127</td>
</tr>
<tr>
<td>L-tyrosine</td>
<td>128</td>
</tr>
<tr>
<td>NADH</td>
<td>129</td>
</tr>
<tr>
<td>amygdalin</td>
<td>130</td>
</tr>
<tr>
<td>hypoxanthine</td>
<td>131</td>
</tr>
<tr>
<td>inosine</td>
<td>132</td>
</tr>
<tr>
<td>hydrogen peroxide</td>
<td>133-134</td>
</tr>
<tr>
<td>alanine</td>
<td>119</td>
</tr>
<tr>
<td>uric acid</td>
<td>135-136</td>
</tr>
<tr>
<td>L-glutamate</td>
<td>137</td>
</tr>
<tr>
<td>glyoxylate</td>
<td>138</td>
</tr>
<tr>
<td>L-ascorbic acid</td>
<td>139</td>
</tr>
<tr>
<td>ADP</td>
<td>140</td>
</tr>
<tr>
<td>cholesterol</td>
<td>3, 141, 142-145</td>
</tr>
<tr>
<td>p-cresol</td>
<td>146</td>
</tr>
<tr>
<td>lactic acid</td>
<td>147</td>
</tr>
<tr>
<td>glucosinolates</td>
<td>148</td>
</tr>
<tr>
<td>cyanide</td>
<td>149</td>
</tr>
<tr>
<td>creatine</td>
<td>150</td>
</tr>
<tr>
<td>aspartame</td>
<td>151</td>
</tr>
<tr>
<td>glucose</td>
<td>152-155</td>
</tr>
<tr>
<td>L-glutamate</td>
<td>156</td>
</tr>
<tr>
<td>L-malate</td>
<td>157</td>
</tr>
<tr>
<td>phenols</td>
<td>158-160</td>
</tr>
<tr>
<td>phosphate</td>
<td>161</td>
</tr>
<tr>
<td>o-Alanine</td>
<td>162</td>
</tr>
<tr>
<td>Atrazine</td>
<td>163</td>
</tr>
<tr>
<td>dopamine</td>
<td>164</td>
</tr>
<tr>
<td>sulphite</td>
<td>104</td>
</tr>
</tbody>
</table>

Adapted from [105,223].
The equipment necessary for amperometric enzyme electrode includes a cell with a magnetic stirrer, sensor body (which comprises working reference and counter electrodes); a computer and potentiostat with amperometric read out \[^{165,166}\]. The amperometric detector is the most widely used electrochemical mode of detection that is used in conjunction with an enzyme electrode. To date, mainly biological substances have been analysed by enzyme electrodes \[^{167}\].

### 1.4.2 Potentiometric Detectors

A two-electrode system is used in potentiometry, where an interfacial potential between the species and electrode is established. The Nernst equation provides a simple relationship between the concentration of the corresponding ionic species and the relative potential of the electrode. Equation 1.7 illustrates this relationship. The measurement of the potential of the reversible electrode permits calculation of the activity of the component in the solution \[^{168}\].

Detecting electrodes for potentiometric systems include redox electrodes, ammonia gas sensor, carbon dioxide gas sensor, pH electrode, monovalent anion and cation selective electrodes \[^{169}\].

\[
E_{\text{cell}} = E^0 \pm \frac{RT}{nF} \ln \left[ \text{analyte} \right] \tag{1.7}
\]

where: \(E_{\text{cell}}\) = the e.m.f of the cell; \(E^0\) = Standard e.m.f of the cell; \(R\) = universal gas constant (8.314 J K\(^{-1}\) mol\(^{-1}\)); \(T\) = temperature in degrees
Kelvin; n = number of electrons transferred for each formula unit of the reaction and F = Faraday's constant (96485 C mol⁻¹).

1.4.3 Voltammetric, Conductometric and Other Detectors

A three-electrode system is employed (working, reference and auxiliary electrodes). This involves simultaneous measurement of the current as the potential of the working electrode is varied. Hence, the concentration of the analyte is proportional to the oxidised or reduced peak current of the species. The analyte cholesterol has been repeatedly analysed using the technique of cyclic voltammetry [170]. Cyclic voltammetry can provide qualitative and quantitative information about electrochemically coupled enzymatic reactions, upon which mediated amperometric biosensors are based.

Coulometry is similar to amperometry in that it involves the use of a constant applied potential. However this technique differs in that it is the total charge transfer that is monitored instead of the current. This is an absolute method, requiring no calibration since the total charge passed is independent of kinetics. To be sure that all of an electroactive species generated by an analytical reaction is consumed in a reasonable time, it is necessary to carry out the assay in a small and accurately known volume [54,170]. This has been achieved most conveniently in the capillary fill device.
Conductometry is the measurement of resistivity and is used frequently for analytical applications. Renewed interest has emerged in the practical applications of conductometry. Recent developments in the electronic field has resulted in greater precision in automated conductometric instrumentation [171,172]. Penicillin has been determined by a PPy-based enzyme electrode with conductometric measurement [173]. The conductivity of the PPy film appeared to be sensitive to the pH of the solution. Hence a PPy-coated electrode can serve as a pH sensitive transducer for enzyme electrodes.

Piezoelectric transducers are conventionally used to measure small masses of materials in applications, such as the vacuum deposition of metals. These devices are able to generate and transmit acoustic waves in a frequency dependent manner. Changes of mass or density at the crystal surface change the resonant frequency enabling them to be used to monitor, for example, the binding of biological molecules [54,174]. Bulk wave devices operate by transmitting a wave from one side of the crystal to the other. Surface acoustic wave (SAW) sensors transmit waves along a single crystal face. The basic concepts of this approach are relatively simple, but the use of piezoelectric crystals in liquids is relatively new. Presently piezoelectric crystal biosensors are being utilised for environmental analysis [175] and more recently reusable piezoelectric crystals have been developed for the analysis of human serum [176].

Other methods such as optical detection and ion-sensitive field-effect transistor (ISFET) have also been used in the construction of enzyme electrodes. Fibre optic detectors have been used for the analysis of
cholesterol \textsuperscript{24}, glucose \textsuperscript{177-179}, lactate \textsuperscript{180}, ATP \textsuperscript{181} and NADH \textsuperscript{181}. Silicon ISFET have also been developed to fabricate urea \textsuperscript{182} and penicillin \textsuperscript{183} biosensors. Recent trends have also seen a move away from the more traditional electrochemical detectors to investigate these newly developed methods.

1.5 IMMOLISATION METHODS

A very important step in the fabrication of a biosensor is the need to immobilise the biological component on the transducer. Immobilisation is the physical confinement or the localisation of enzyme molecules \textsuperscript{188}. There are three main reasons for immobilising enzymes. Firstly, it offers an immense operational advantage over freely mobile enzymes; secondly, immobilised enzyme may exhibit altered physical properties or chemical properties; and finally it may serve as a model system for natural in-vivo membrane bound enzymes \textsuperscript{184}. There are also a number of operational advantages associated with immobilised enzymes such as controlled product format, reusability, possibility of batch or continuous operational modes, rapid termination of reactions, greater variety of engineering designs for continuous processes and greater efficiency in consecutive multi-step reactions \textsuperscript{196}.

The properties and the characteristics of the biosensing layer are extremely important in the design of the biosensor, especially in terms of time-dependant reliability, since they are repeatedly in contact with the sample \textsuperscript{54}. For the repeated use of enzyme molecules in biosensors,
numerous methods for immobilising the molecules to the support materials have been developed \cite{46}. Soluble enzyme molecules are readily dispersed in the solution and have complete freedom of movement. Enzyme immobilisation is a method that has been designed to greatly restrict the freedom of movement of the enzyme molecules. In general the immobilisation material may function purely as a support \cite{184}. However, it could also be concerned with the mediation of the signal transduction mechanism associated with the analyte \cite{185}. Various immobilisation methods can be chosen for the preparation of the sensing layer. There are two main categories of layers. The first are pre-formed artificial membranes bearing functional groups which can be further activated and coupled to appropriate enzymes. The second category includes polymer matrices in which the enzyme molecules are trapped and can be used for direct coating onto the electrode \cite{48}.

The immobilisation of enzymes has some unique advantages for their use in analytical chemistry, such as: \cite{46} stabilisation of the enzyme; ease of separation of the enzyme-carrier complex from the sample, i.e. the latter is not contaminated by the enzyme preparation; maintaining a stable and largely constant enzyme activity, rendering the enzyme an integral part of the analytical instrument.

The selection of the support material and the method of immobilisation, is made by weighing the various characteristics and required features of the enzyme/cell application against the properties, limitations and characteristics of the combined immobilisation/support \cite{184}. There are also a number of practical aspects that should be considered before undertaking any experimental work. This ensures that the final enzyme
and/or cell preparation is fit for the planned purpose or application and will operate at optimum effectiveness \(^{186-188}\). The first consideration is to decide on the support material and then the method of immobilisation, taking into account the intended use and application. Some points to consider when making a decision are listed in Table 1.5 \(^{184}\). Of particular interest among these are physical, chemical, stability and resistance properties.

Available methods for immobilisation of enzymes can be classified as physical and chemical, but may also involve the use of both methods \(^{46}\). The common immobilisation methods are described below.

### 1.5.1 Adsorption

The adsorption of enzyme molecules onto support materials that are insoluble in water is the simplest method of immobilisation \(^{46,49,184}\). It involves the reversible surface interaction between the enzyme/cell and the support material \(^{189,190}\), as illustrated in Figure 1.5.

![Figure 1.5: Schematic of adsorption. Adapted from \(^{31,46,184}\).](image-url)
Table 1.5:

Fundamental considerations in selecting a support and method of immobilisation

<table>
<thead>
<tr>
<th>Property</th>
<th>Points for Consideration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physical</td>
<td>Strength, non compression of particles, available surface area, shape/form (beads/sheets/fibres), degree of porosity, pore volume, permeability, density, space for increased biomass, flow rate and pressure drop</td>
</tr>
<tr>
<td>Chemical</td>
<td>Hydrophilicity (water binding by the support), inertness towards enzyme/cell, available functional groups for modification and regeneration/reuse of support</td>
</tr>
<tr>
<td>Stability</td>
<td>Storage, residual enzyme activity, cell productivity, regeneration of enzyme activity, maintenance of cell viability, and mechanical stability of support material</td>
</tr>
<tr>
<td>Resistance</td>
<td>Bacterial/fungal attack, disruption by chemicals, pH, temperature, organic solvents, proteases, and cell defence mechanisms (proteins/cells)</td>
</tr>
<tr>
<td>Safety</td>
<td>Biocompatibility (invokes an immune response), toxicity of component reagents, health and safety process for workers and end product users, specification of immobilised preparation (GRAS list requirements for FDA approval) for food, pharmaceutical and medical applications</td>
</tr>
<tr>
<td>Economic</td>
<td>Availability and cost of support, chemicals, special equipment, reagents, technical skills required, environmental impact, industrial-scale chemical preparation, feasibility for scale-up, continuous processing, effective working life, reusable support and CRL or zero contamination (enzyme/cell-free product)</td>
</tr>
<tr>
<td>Reaction</td>
<td>Flow rate, enzyme/cell loading and catalytic productivity, reaction kinetics, side reactions, multiple enzyme and/or cell systems, batch, CSTR, PBR, FBR, ALR, and so on; diffusion limitations on mass transfer of cofactors, substrates and products</td>
</tr>
</tbody>
</table>

The procedure involves mixing or bringing into contact an aqueous solution of the enzyme molecules with a support material which has adsorption properties. The support may also be referred to as an active support material. This is allowed to occur for a defined period of “incubation” or time \[^{[46,184]}\]. Thereafter the immobilised material is collected and any non-bound molecules are removed by extensive washing \[^{[191]}\].

Active charcoal, silica gel, clay aluminium oxide, anionic and cationic exchange resins, ceramic and porous glass are currently being used as active support materials \[^{[46]}\]. The active support material must exhibit a high affinity and capacity for the enzyme and neither the reaction products nor the inhibitors of the enzyme should be adsorbed. The enzyme on the other hand must remain active in the adsorbed state \[^{[46,191]}\].

The forces involved are mostly electrostatic. Among these are Van der Waals forces, ionic and hydrogen bonding interactions of which hydrophobic bonding can be quite significant. Although the forces are termed relatively weak, they are large enough to enable reasonable binding. A simple example to illustrate this is the use of a positive support for immobilisation of yeast cells that have a substantially negatively charged surface chemistry \[^{[31]}\]. The method has both advantages and disadvantages, described below \[^{[184]}\].

\textit{Advantages of Adsorption}:

1. Little if any damage to enzymes/cells;
2. Simple, cheap and quick to obtain immobilisation;
3. Reversible, to allow regeneration with fresh enzymes/cells;
4. No chemical changes to support or enzyme/cell.

**Disadvantages of Adsorption:**

1. Leakage of enzymes/cells from support or contamination of product;
2. Non-specific binding;
3. Overloading of the support; and
4. Steric hindrance of the support.

Perhaps the most significant disadvantage is the leakage of the enzyme from the support\textsuperscript{[192]}. Desorption can occur under several circumstances and environmental changes in pH, temperature, and ionic strength\textsuperscript{[192]}. Sometimes cells/enzyme, firmly adsorbed may be readily desorbed during a reaction as a result of substrate binding, binding of contaminants present in the substrate product formation or other conditions leading to a change in protein conformation. Other factors which may also lead to desorption are physical factors, such as flow rate, bubble agitation and particle-particle or the scouring effect of the particulate materials on the vessel walls\textsuperscript{[192]}. Adsorption of COD and CE onto glass beads has been used previously for the development of a biosensor for the determination of total serum cholesterol\textsuperscript{[41]}\textsuperscript{.} Amperometric FIA was used as the mode of detection\textsuperscript{[41]}. A COD-CE enzyme electrode was also developed for the successful determination of free and total cholesterol via adsorption, involving laponite clay additives\textsuperscript{[42]}. Adsorption of COD onto glass was also employed for cholesterol assays in a thermistor device\textsuperscript{[26]}.
1.5.2 Covalent Bonding or Covalent Coupling

This method of immobilisation involves the formation of a covalent bond between the enzyme/cell and a support material \[^{46,190,194,195}\] as illustrated in Figure 1.6. The bond is usually formed between the functional groups present on the surface of the support material and functional groups belonging to amino acid residues on the enzyme surface. There are several amino acid functional groups that are suitable for participation in covalent bond formation. The groups that are most often involved include the amino group (NH\(_2\)) of lysine and arginine, the carboxyl group (CO\(_2\)H) of aspartic acid and glutamic acid, the hydroxyl group (OH) of serine or threonine and the sulphydryl group (SH) of cysteine \[^{46,196}\].

There is an extensive range of support materials available for covalent bonding. This demonstrates the versatility of the method. Hence the advantages and disadvantages of a support must be taken into consideration when deciding on possible procedures for a given enzyme immobilisation \[^{197,198}\]. Research has shown that of all the factors that should be taken into account, hydrophilicity is the most important factor for maintaining enzyme activity in a support \[^{199}\]. The sugar residues in these polymers contain hydroxyl groups, which are ideal functional groups for chemical activation to provide covalent bond formation. Also hydroxyl groups form hydrogen bonds with water molecules and thereby create an aqueous (hydrophilic) environment in the support. Consequently polysaccharide polymers, such as cellulose, dextran and agarose derivatives, which are very hydrophilic, are very popular support materials for enzyme immobilisation by covalent bonding.
One slight disadvantage of polysaccharide supports is that they are susceptible to microbial or fungal disintegration and organic solvents can cause shrinkage of the gels. Hence, inorganic materials, such as porous glass and porous silica have also become popular. These materials are not only durable, but are also resistant to microbial disintegration or solvent distortion. However, these two supports are not as hydrophilic as the polysaccharide materials \cite{184}. Other groups of supports include high molecular weight proteins (collagen, gelatin and albumin) as well as synthetic polymers (PVC and ion exchange resins) \cite{46,184}.

There are many reaction procedures for coupling an enzyme and a support in a covalent bond. Immobilisation can be conducted in three steps: \cite{46,184}

1. Activation of the functional groups on the support material by a specific reagent;

2. the enzyme is added in a coupling reaction to form a covalent bond with the support material; and

3. removal of adsorbed enzymes.

This method has been used in the determination of both free \cite{21,38} and total \cite{39} cholesterol. In the case of the former COD was immobilised onto a pre-activated collagen membrane and for the latter both COD and CE were immobilised on a pre-activated collagen membrane \cite{21,38,39}.
1.5.3 Entrapment

Immobilisation by entrapment has been described as being as mild a procedure as adsorption, because the enzyme molecules are not covalently bound to the matrix, the membrane or to each other \cite{46}, as shown in Figure 1.6. Entrapment differs from adsorption and covalent bonding because the enzyme molecules are free in solution. However, their movement is restricted by the lattice structure of the gel in which they are immobilised \cite{200,201}. Controlling the porosity of the gel lattice is important. The matrix structure must be tight enough to prevent leakage of the enzymes/cells, yet at the same time allow free movement of the substrate and product. Inevitably, the support will act as a barrier to mass transfer. This could have serious implications for the reaction kinetics. However, the useful advantage is that harmful cells, proteins and enzymes are prevented from interacting with the immobilised biocatalyst \cite{202,203}.

**Figure 1.6:** Schematic of covalent binding. Adapted from \cite{31,46,184}.
There are several methods of entrapment, which include: [184]

1. **Ionotropic gelation of macromolecules with multivalent cations (eg. alginate);**

   Entrapment can be achieved by mixing an enzyme with a polyionic polymer material and then cross-linking the polymer with multivalent cations in an ion-exchange reaction to form a lattice that traps the enzymes/cells [36,184].

2. **Temperature-induced gelation (eg. agarose, gelatin);**

   Temperature change is a simple method of gelation by phase transition using 1-4% solutions of agarose or gelatin [31]. However, the gels formed are soft and unstable. A major development in this area of research has been the introduction of κ-carrageenan polymers that are able to form gels by ionotropic gelation and by temperature-induced phase transition [184]. This has introduced a greater degree of flexibility in gelation systems for immobilisation.

3. **Organic polymerisation by chemical/photochemical reaction (eg. polyacrylamide); and**

   It is possible to mix the enzyme with chemical monomers that are then polymerised to form a cross-linked polymeric network, trapping the enzyme in the interstitial spaces of the lattice [184]. This method is more widely used than methods 1 and 2 described above, and there are now a number of acrylic monomers available for the formation of hydrophilic
copolymers. In addition to the monomer, a cross-linking agent is added during the polymerisation step to form cross-linkages between the polymer chains and to help create a three-dimensional network lattice. The amounts of the monomer and the cross-linking agent determine the pore size of the gel and its mechanical properties. Hence, it is possible to influence the lattice structure by varying these concentrations. The resulting polymer can then be broken up into particles of a desired size or polymerisation can be arranged to form beads of defined size.

4. Precipitation from an immiscible solvent (eg. polystyrene).

This method is limited to highly stable/previous-stabilised enzymes or non-living cells \(^{[184]}\). Precipitation occurs by phase separation rather than by chemical reaction, but does not bring the cells/enzymes into contact with a water-miscible organic solvent and most cells are not tolerant of such solvents.

The reported use of this immobilisation method for the development of biosensors for cholesterol measurement involves the entrapment of COD in PPy films \(^{[3]}\) and entrapment of COD in overoxidised PPy films \(^{[42]}\). COD and CE have been immobilised on agarose gel for the assay of total cholesterol \(^{[26]}\). This method has also has been used previously for the successful entrapment of the enzyme L-lyseine oxidase in a gelatin support \(^{[31]}\).
1.5.4 Encapsulation

Encapsulation of enzymes or cells can be achieved by enveloping the biological components within various forms of semipermeable membranes \cite{204-206}, as illustrated in Figure 1.8. It is similar to entrapment in that the enzymes or cells are free to move, but they are restricted in space. Large proteins or enzymes can pass neither into nor out of the capsule. However, small substrates and products can pass freely over the semipermeable membrane. Several materials have been used to construct these microcapsules varying from 10-100 μm in diameter; for example, nylon and cellulose nitrate have been popular choices \cite{184}. A major disadvantage that is associated with diffusion is that the membrane could rupture if there is a very rapid accumulation of products. However, a distinct advantage of encapsulation is co-immobilisation. Cells and/or
enzymes may be immobilised in any desired combination to suit particular applications.

**Figure 1.8:** Schematic of encapsulation. Adapted from [31,36,184].

1.5.5 Cross-linking

This method of immobilisation is support-free and involves joining the enzymes or cells to each other to form a large three-dimensional rather complex structure, as illustrated in Figure 1.9. There are two main methods of cross-linking: [184]

(a) **Chemical Cross-linking**

This usually involves covalent bond formation between the cells by means of a bi/multifunctional reagent, such as glutaraldehyde, bisisocyanate derivatives and bisdiazobenzidine [36,184,207]. A major limiting factor in
applying this method to living cells and many enzymes, is the toxicity of the above mentioned reagents. Functionally inert proteins, such as albumin and gelatin have been used to provide additional protein molecules as spacers to minimise the close proximity problems that can be caused by cross-linking a single enzyme \[^{31,36}\]. The advantages of this method include simplicity of the procedure and strong chemical binding of the biomolecules. Furthermore, the choice of the degree of cross-linking permits the physical properties and the particle size to be influenced. The main drawback is the possibility of activity losses due to chemical alterations of the catalytically essential sites of the protein \[^{31}\].

\[(b) \quad \textit{Physical Cross-linking}\]

This method is well known in the biotechnology industry where cells are physically cross-linked by flocculation, which leads to high cell densities \[^{207}\]. Flocculating agents, such as polyamines, polyethyleneimine, polystyrene sulfonates and various phosphates, have been used extensively and are well characterised. This method is rarely used, as the sole means for immobilisation because the absence of mechanical properties and poor stability are severe limitations. It is more often used to enhance other methods of immobilisation, normally by reducing cell leakage in other systems \[^{184}\].

Yao et. al. \[^{44}\] have reported on the successful, simultaneous assay of free and total cholesterol in blood serum by FIA. The enzyme electrode is prepared by cross-linking peroxidase with BSA-GLA on a gold sheet and
the use of immobilised COD-CE enzymes for the development of a cholesterol biosensor for the accurate determination of total cholesterol. Dong et. al. [36] have fabricated a cholesterol biosensor where COD is immobilised onto a palladium (Pd) dispersed electrode by cross-linking with GLA, and a layer of o-PPD was electropolymerised on the COD-GLA layer.

**Figure 1.9:** Schematic of cross-linking. Adapted from [31,46,184].

1.6 MEMBRANES FOR ENZYME IMMOBILISATION

There are several membranes that have been used for immobilisation of enzymes. Some of these are discussed below.
1.6.1 Conducting Polymer Films

Conducting polymer films are widely used often to enhance electron transfer characteristics \[^{113}\]. They are capable of conducting electronic charge. Ivaska \[^{208}\] states in his review on conducting polymer films that PPy is one of the six most important polymer films. PPy and its derivatives have gained considerable interest in biosensor development due to their versatile applicability and the wide variety of molecular (redox) species covalently linked to the PPy group \[^{209,210}\]. Also listed amongst the six are polythiophene, poly-3-alkylthiophenes, poly(p-phenylene) and polyaniline \[^{113}\]. Most of the electrochemically deposited polymer films used for the biomolecule immobilisation are conducting polymer films \[^{113,211}\]. The advantage of these polymers is that the films can be prepared easily via electropolymerisation in a rapid one-step procedure. Furthermore, the method enables exact control of film thickness of the polymer, layer based on the measurement of the electrical charge passed during the electropolymerisation. Conducting polymer films can be divided into two categories: electronically conducting polymers, such as polyacetylene described by Soliton theory \[^{212,213}\] and band theory; and redox conducting polymers which are the second category of conducting polymer films, described by site hopping phenomena \[^{214}\]. The polymers may be produced either chemically or through electrochemical polymerisation. Conducting polymer films are incorporated in the biosensor to facilitate electron transfer from a biocomponent to the electrode surface, for the incorporation of mediators and in the immobilisation of enzymes \[^{113}\]. A
limitation of conducting films is that they may interact with interferents or oxygen to produce deleterious effects.

1.6.2 Non-Conducting Polymer Films

Non-conducting polymer films have also been incorporated into biosensors for the following reasons; to prevent interferences, to prevent fouling of the electrode by substances, such as proteins, to immobilise the biocomponent and to entrap the mediator so that it does not leach away [215]. When non-conducting polymer films are formed by electropolymerisation techniques, they show characteristics of molecular self-assembly [113]. The films are self-regulating and there is a guarantee of complete electrode coverage regardless of size or shape. Uniformity in the film is maintained because the film only grows thick enough to become an insulator. Polymerisation continues until the surface is completely covered. This is signalled by the current decreasing to a minimum, because the monomer cannot penetrate the polymer film. The typical film thickness for non-conducting polymer films is approximately 10 nm. Earlier work in this area used non-conducting polymer films to prevent interferences from reaching the electrode surface.

1.6.3 Composite Polymer Films

Sometimes when two or more different types of polymers are blended together to form a composite membrane, the properties of the "new" blended membrane surpass those of the individual polymers.
Koopal et. al. [216] reported on the use of a mixture of agarose solution and latex suspension to create a porous membrane on a platinum coated glassy carbon disk electrode. Electropolymerisation was performed galvanostatically in aqueous solution. The immobilisation of glucose was carried out by agitating composite membranes in a solution of GOD. Finally, the amperometric response to the glucose was achieved over a range between 1 and 60 mM.

1.6.4 Conductive Polypyrrole Films and Biosensors

The use of conductive polymer films has become very attractive for sensor development. The use of PPy as a sensing component or as a medium for the immobilisation of biomolecules, such as enzymes or other important biological proteins is becoming increasingly common [217]. The most attractive feature of PPy is its high stability at elevated temperatures. There is also the possibility of much wider analytical utilisation of biosensors in the presence of oxygen. To date this has been limited by the instability of enzymes.

PPy films provide a multi-layered, dynamic polymeric coating, which has a three-dimensional reaction zone at the electrode surface. It is on this surface that various chemical events, such as ion exchange, complexation, precipitation and various enzyme reactions can be performed [217]. PPy is a very versatile conductive polymer film and enables the incorporation of a wide variety of counter ions into the polymer. However the counter ion does influence the physical properties of the film, including morphology,
conductivity, adhesion and mechanical strength \cite{218}. The performance of PPy is superior when compared to other heteroaromatic polymers. This is because PPy may be readily synthesised from a range of media \cite{219-221}. Furthermore it is convenient to directly incorporate a range of biologically active proteins from aqueous solutions during polymerisation of pyrrole to PPy \cite{113}.

1.7 FABRICATION OF POLYMER FILMS

There are several methods of polymer film fabrication; solvent casting, spin coating, adsorption and electropolymerisation. Electropolymerisation is by far the most widely used and recognised technique because of the excellent characteristics of the resulting polymer films.

1.7.1 Solvent Casting

Previously, this method was used extensively in the application of films to electrodes. The method involves a polymer film being formed by the evaporation of a polymer solution that is placed on the surface of the electrode \cite{113}. The major limitation of this method is that it is difficult to control precisely. It is also difficult to obtain complete and uniform coverage of an electrode, especially when aiming for thin film formation. Solvent casting is also limited to two dimensional or simple surfaces.

1.7.2 Spin Coating

This method is used extensively in the electronics industry. Spin coating is a method for forming films of controllable and uniform thickness, on a
substrate such as an electrode surface [222]. The procedure involves dissolving the polymer in an appropriate solvent and having microliter quantities of the polymer solution being dropped onto the electrode, which is mechanically attached to a spinner that rotates at high speeds such as 4000 rpm. The film is air dried as it continues to spin for several minutes. Usually, numerous monolayers must be applied to obtain a film-covered electrode that is free from pinholes [113].

1.7.3 Adsorption of Polymer Film onto an Electrode

This is a very simple method of film formation. It is difficult to control the film thickness with this method. Recently however, the method has been modified so that it precedes electropolymerisation. In this way, the process of adsorption enhances the technique of electropolymerisation and produces polymer film of better quality, with more of the desired characteristics for detection.

1.7.4 Electropolymerisation

When the transfer of electrons at the electrode-solution interface that results from the passage of the current through the solution is manifested as oxidation or reduction reactions, in which reactive intermediates are produced, chain polymerisation may result [222]. One electron per chain may initiate free radical or ionic polymerisation. Electropolymerisation may also be achieved if initial electron transfer takes place, that permits coupling reactions to occur that lead to additional chain growth as additional electrons are transferred [113]. In addition, condensation
reactions in which monomer molecules combine with a corresponding loss of simple molecules such as water, can also be electrochemically initiated. Faraday's Law governs electropolymerisation \cite{222}. The rate at which polymer formation occurs is determined by the current flow. The amount of polymer generated is governed by the total amount of charge transferred. For surface films, the total charge transferred determines the thickness of the layer of polymer formed.

Typical electrode materials for electropolymerisation include glassy carbon, platinum or gold \cite{113,211}. Good films with acceptable adherence are obtained on gold and platinum. However, the potential use of these materials is not as high as that of glassy carbon. With gold and platinum electrodes the film is often stripped by the evolution of hydrogen or oxygen \cite{113}. There are other electrode materials like indium-tin, oxide coated glass, titanium, aluminium, mild steel and brass. Under certain conditions films may be produced on brass however poor films are produced on aluminium \cite{211}.

Polymer film formation via electropolymerisation can be performed using potential cycling methods, galvanostatic techniques, fixed potential techniques and pulsed potential approaches.

The use of electropolymerisation to prepare films on microelectrodes and microarray electrodes is a practical approach to direct film formation onto small or irregularly shaped electrodes \cite{223}. The approach is especially attractive for microarray electrodes in particular, because the
electropolymerised film is self-regulating, possesses uniform thickness and covers the electrode evenly regardless of shape or size [223].

Specific advantages with using electropolymerisation are:

1. its ability to coat very small and irregularly shaped objects with a polymeric film;
2. it is also possible to control the film thickness based upon the amount of charge passed (conducting films) or by self-regulation (non-conducting films);
3. both the polymerisation rate and the nature of the film may be influenced via the applied potential that is used during electropolymerisation; and
4. electropolymerised films have been successfully employed in the blockage of interferences that would otherwise undergo oxidation, at the electrode surface as well as the prevention of fouling of the electrodes [211].

1.8 AIMS AND OBJECTIVES

The aim of this study is to develop sensitive, accurate and reliable cholesterol biosensors for the determination of free and total cholesterol. This will involve the optimisation of various solution and instrumental parameters.

The specific objectives include:
(a) investigation of enzyme immobilisation methods, such as entrapment by electropolymerisation and cross-linking with BSA and GLA;

(b) comparison of amperometric and potentiometric modes of detection;

(c) the use of mono and bi-layer designs will be considered for improving the performance of the biosensor for cholesterol;

(d) testing the response of the biosensor by performing recovery studies and

(e) comparison of cholesterol biosensor response to samples pre-analysed by a clinical method.
CHAPTER 2

DEVELOPMENT OF A
POLYPYRROLE-BASED
CHOLESTEROL OXIDASE
BIOSENSOR FOR
CHOLESTEROL
CHAPTER 2

DEVELOPMENT OF A POLYPYRROLE-BASED
CHOLESTEROL OXIDASE BIOSENSOR FOR
CHOLESTEROL

2.1 INTRODUCTION

Several approaches have been employed for the development of biosensors for cholesterol. Many have focused on the immobilisation of COD on different surfaces, such as on screen-printed carbon electrodes\textsuperscript{[4]}, laponite clay additives\textsuperscript{[192]}, pre-activated collagen membrane\textsuperscript{[21,38,39]} and pre-activated nylon membranes\textsuperscript{[24]}. However, with most of these methods, the activity of the enzyme was not constant throughout the experiment possibly due to leaching or de-activation. Other reported methods of COD immobilisation include chemical cross-linking\textsuperscript{[44,136]}, covalent bonding\textsuperscript{[21,38,39]}, entrapment\textsuperscript{[3]} and encapsulation\textsuperscript{[184]}. More accurate and reliable results have been achieved by the cross-linking of COD onto a Pd dispersed electrode with GLA as the cross-linking agent\textsuperscript{[36]}. Another approach for the immobilisation of COD is by entrapment of the enzyme in a PPy film\textsuperscript{[3]}. The COD is present in the monomer solution and is incorporated into the film during galvanostatic film formation on an electrode surface\textsuperscript{[3,222-224]}. The use of conductive PPy has become more common in recent years for the development of cholesterol biosensors because of the distinct advantages of the film\textsuperscript{[226]}. These include: ease of polymerisation and better electrical properties due to PPy conductivity.
The polymerisation procedure is fast, inexpensive and fresh polymer can be readily produced at room temperature [113]. In 1992 Dong et. al. [36] reported that the main disadvantage of cholesterol biosensor fabricated by electropolymerisation of pyrrole is the limited ability to control the enzyme loading in the polymer film. Although COD was entrapped in the polymer film, the current responses to cholesterol were very small. This was because only a small proportion of the enzyme present in the monomer solution was entrapped into the polymer film. This method is not cost-effective because of its ability to immobilise only small amounts of the very expensive enzymes. Another disadvantage associated with the measurement of cholesterol using a PPy based COD biosensor is the suppression of the biosensor response [3]. This is caused by the presence of electroactive interferents, namely ascorbic and uric acid [34] in biological samples.

As there is still considerable interest in the availability of simple, rapid and reproducible methods for the production of cholesterol sensors [3], the use of electropolymerisation of pyrrole or alternative methods for immobilisation of COD, and the application of the resulting biosensors still need to be investigated in more detail.

It has also been reported that although a direct electron transfer is possible between an electrode and an electropolymerised PPy film, sensitivity to hydrogen peroxide is apparently low and decreases rapidly [227]. A way of overcoming this problem is by the use of electron mediators, which enhance the rate at which electron transfer between enzymes and the electrode occurs [33]. Ferrocene derivatives [228], quinone [229] and
ferrocyanide derivatives \cite{230} are all commonly used as mediators in the development of cholesterol biosensors.

In this chapter, an electrochemical approach employed for the development of a PPy-based cholesterol biosensor is described. This involves co-incorporation of COD and \([\text{K}_4\text{Fe(CN)}_6]\) as a mediator into a PPy film by galvanostatic polymerisation. Factors, such as effect of applied potential \((E_{\text{app}})\), polymerisation time, current density, pyrrole concentration, COD concentration and the effect of \([\text{K}_4\text{Fe(CN)}_6]\) concentration, were investigated to obtain optimum response for cholesterol with the biosensor. The use of the biosensor in both amperometric and potentiometric modes will be considered for establishing the more effective detection mode.

\section{2.2 EXPERIMENTAL}

\subsection{2.2.1 Laboratory Conditions}

All experiments were carried out under laboratory conditions and a constant room temperature of 20 °C.

\subsection{2.2.2 Instrumentation}

A potentistat/galvanostat designed and built at the University of Western Sydney was employed for all measurements. This instrument was used in galvanostatic mode for the electropolymerisation, as well as for potentiometric determination and potentiostatic mode for amperometric
detection of cholesterol. A MacLab system (AD Instruments Pty. Ltd) attached to a BAS Voltammograph CV-27 was used to characterise the PPy-COD films by cyclic voltammetry. The potentiostat was connected to a computer controller (AMD-K6 – 400 mHz Celeron processor, 32 MB RAM, 8 Gigabyte HD, Hansol Monitor and windows ’98 keyboard and mouse) and a Brother HL-12707 networked laser printer. The solution was stirred when necessary with a Sybron Thermolyne (model S-17410). Potentiometric measurements were carried out with a two-electrode electrochemical cell, consisting of a working platinum electrode (onto which the enzyme was immobilised) and silver/silver chloride reference electrode.

Amperometric measurements were carried out with a three-electrode electrochemical cell system, consisting of a working platinum electrode, a silver/silver chloride (Ag/AgCl) reference electrode and a platinum wire as the auxiliary electrode.

2.2.3 Glassware

Prior to the initial measurement, all the glassware and polyethylene cells that were used for solution preparations and measurements, respectively, were soaked in an acid bath (1 %v/v HCl: 1 %v/v HNO₃) for at least one week. Then after each use, the glassware and cells were washed initially with detergent and then soaked in an acid bath (1 %v/v HCl: 1 %v/v HNO₃) at least overnight. Prior to use, each item was rinsed several times with fresh Mill-Q water.
2.2.4 Chemicals and Standard Solutions

All chemicals were of analytical grade, unless otherwise stated. All solutions were prepared using Milli-Q grade water (18 Ω cm⁻¹). Cholesterol, water-soluble cholesterol, cholesteryl undec-10-enoate and cholesterol oxidase (EC 1.1.3.6) from cellulosmonas were purchased from the Sigma-Aldrich Chemical Company. Cholesterol was also purchased from the AJAX chemical company. The water-soluble cholesterol was stored in the freezer until required. The COD solution was divided into 20 μL aliquots and stored in the freezer in capped and sealed Eppendorf cells, until needed. Phosphate buffer stock solution (0.5 M, pH 7.0) was prepared by neutralising orthophosphoric acid with sodium hydroxide. This was stored in the fridge and diluted as necessary. A 0.1 M sodium nitrate (NaNO₃) solution was prepared by dissolving an appropriate amount (2.1250 g) of NaNO₃ in Milli-Q water. The volume was then adjusted to 250 mL. The pyrrole was distilled under vacuum at 130 °C prior to use. Pyrrole is both light and heat sensitive. Hence, it was stored in an aluminium foil-covered sample tube in the freezer to prevent UV degradation. A stock solution of 0.25 M [K₄Fe(CN)₆] was prepared by dissolving 1.0060 g of [K₄Fe(CN)₆] in Milli-Q water. The volume was then adjusted to 10 mL. The volumetric flask was then placed in an ultrasonic-bath, briefly until any remaining crystals dissolved. [K₄Fe(CN)₆] tends to undergo UV degradation and oxidation by dissolved oxygen. Hence, to minimise the effect of UV degradation the solution was transferred to an aluminium foil-covered sample tube and was stored in the refrigerator at ~4 °C until required.
2.2.5 Procedures

(a) Electrode Preparation

The platinum working-electrode was polished with 320 μM aluminium oxide, on a soft polishing pad, to remove any previous film and then finally polished with 5 μM aluminium oxide. The electrode surface was thoroughly washed with Milli-Q water rinsed under a stream of acetone and finally rinsed thoroughly with Milli-Q water to remove any of the remaining aluminium oxide. The electrode was dried with fibre free tissue paper and fixed onto a retort stand for the next step.

(b) Electropolymerisation of PPy-COD-[Fe(CN)_6]^{4+} Film

The galvanostatic electropolymerisation of the PPy film was performed using a three-electrode voltammetric cell. The working electrode was a platinum electrode (0.17 cm²), whilst a platinum wire and a Ag/AgCl (filled with saturated KCl) were used as the auxiliary and reference electrodes respectively. The PPy-COD-[Fe(CN)_6]^{4+} film was formed from a solution containing 0.3 M pyrrole, 25 units of COD and 5 mM [K_4Fe(CN)_6] which had been purged with nitrogen for 10 minutes prior to film formation. A current density of 0.5 mA/cm² was applied for 100 s. After the galvanostatic film formation, the polymer electrode was washed several times under a stream of Milli-Q water to remove any weakly bound COD or [K_4Fe(CN)_6] molecules prior to analysis.
(c) **Cholesterol Standard**

A 5 mM cholesterol standard solution was prepared by dissolving 0.19335 g of cholesterol in 12.8 mL of 2-propanol and 3.85 mL of Triton X-100. Then 0.05 M phosphate buffer (pH 7.0) was added and the volume was adjusted to 100.00 mL. The volumetric flask was then placed in an ultrasonic bath for at least two hours or until all the cholesterol had dissolved and a clear and colourless solution remained. The solution was stored in the refrigerator at ~ 4 °C and was stable for about 7-10 days, and then slowly became turbid. Fresh solution was prepared weekly, or at the first visible sign of turbidity. The cholesterol standard used for the measurement of total cholesterol, contained cholesterol and cholesteryl undec-10-enoate in such amounts that the ratio of free to esterified cholesterol was 30 % (free): 70 % esterified. Owing to the low solubility, this solution was prepared as above in phosphate buffer, with 12.8 mL of 2-propanol and 3.85 mL of Triton X-100.

(d) **Amperometric and Potentiometric Measurement**

Amperometric measurement was performed on a conventional three-electrode system while a two-electrode electrochemical cell was employed for all potentiometric measurements. In both methods the cell contained 20 mL of phosphate buffer (pH 7.0) which was stirred with a magnetic stirrer.

The response of the sensor under different conditions was measured by additions of the standard cholesterol solution.
The minimum detectable amount was determined by continuous additions of cholesterol until a response was obtained. The experiment was repeated three times to confirm the minimum detectable amount obtained. The stability was established for amperometric measurement after waiting for a minimum period of four minutes. In potentiometric measurement the stability was established after waiting for a minimum period of six minutes.

(e) Electrode Storage

When not in use the PPy-COD-[Fe(CN)$_6$]$^{4-}$ electrode was suspended with an o-ring, to avoid damage to the film, in a polyethylene tube containing 0.05 M phosphate buffer (pH 7.0) and stored in the refrigerator.

2.3 RESULTS AND DISCUSSION

2.3.1 Characterisation by Chronopotentiometry and Cyclic Voltammetry

(a) Chronopotentiometry

The conductivity and ease of polymerisation of PPy films can be investigated by chronopotentiometry. The technique involves monitoring the change in potential versus time during constant current polymerisation, which is illustrated by chronopotentiograms. The level of electrical conductivity of a film can be determined by studying the chronopotentiogram. Generally conductive polymer materials have a low initial potential for polymerisation, and the potential either remains low or decreases during the polymerisation [105].
Figure 2.1 shows typical chronopotentiograms obtained for the polymerisation of PPy-NO₃ film (a) and PPy-COD film (b). Figure 2.1 (a) shows that the PPy-NO₃ film had a very low initial potential of 291 mV in the chronopotentiometric measurement. This indicates that the polymer film was easy to polymerise. The potential increased to a maximum of 516 mV then decreased before finally stabilising at a potential of ~480 mV.

Figure 2.1 (b) for the PPy-COD film shows that the initial potential for polymerisation was 384 mV. The potential continued to increase during the film formation, before stabilising at a maximum potential of ~654 mV.

The potential values obtained for the PPy-COD film are higher than that obtained for the PPy-NO₃ film indicating that the PPy-COD film was slightly less conductive than the PPy-NO₃ film. The higher initial and film-formation potential produced for the PPy-COD film indicates the high resistance (therefore low conductivity) of the pyrrole-COD monomer solution. It also indicates that the PPy-COD film was more difficult to polymerise than the PPy-NO₃ film. The macromolecular structure of COD may be inhibiting the free radical polymerisation of pyrrole slightly and hence, decreasing the conductivity of the film.
Figure 2.1:

Characterisation of (a) PPy-NO₃ and (b) PPy-COD Films by Chronopotentiometry:

Conditions for: (a) 0.3 M pyrrole, 0.1 M KNO₃, 0.5 mA/cm² for 100 seconds.

(b) As above except 31.25 units/mL of COD was added instead of KNO₃.
(b) *Cyclic Voltammetry*

The cyclic voltammogram (CV) obtained for a PPy-NO$_3$ film in 0.1 M NaNO$_3$ is illustrated in Figure 2.2 (a). The applied potential was scanned linearly from +400 mV to ~ -800 mV at a scan rate of 50 mV/s. The characteristic oxidation and reduction couple of PPy appeared at approximately -0.1 and -0.15 V versus Ag/AgCl, respectively.

However, when COD was substituted for the nitrate ion the cyclic voltammetric behaviour of the film was altered as shown in Figure 2.2 (b). The characteristic oxidation and reduction couple of PPy appears to have shifted to approximately -0.05 and 0.5 V, respectively, versus Ag/AgCl. The incorporation of the COD into the PPy may have decreased the conductivity of the film and caused the shift in potential.
Figure 2.2:
Characterisation of (a) PPy-NO₃ and (b) PPy-COD Films in 0.1 M NaNO₃ by cyclic voltammetry. Polymerisation conditions were as described in Figure 2.1 and the scan rate was 50 mV/s
2.3.2 Effect of Applied Potential ($E_{app}$)

Figure 2.3 shows the amperometric responses obtained for cholesterol with the variation of the electrode potential between -350 and +700 mV. The response increased as the applied potential became less positive, reaching an optimum value at -200 mV (vs Ag/AgCl). The cholesterol response obtained at this potential had a lower background and was more reproducible. At potentials that were more negative, the cholesterol response decreased considerably and the background was very noisy. This may be due to the increased tendency to reduce oxygen at the more negative potentials\textsuperscript{[170]} as illustrated by equation 2.1:

$$\frac{1}{2} \text{O}_2 + 2\text{H}^+ + 2\text{e}^- \rightarrow \text{H}_2\text{O} \quad (2.1)$$

The reduction of the oxygen may impair the efficiency of the PPy-COD-[Fe(CN)\textsubscript{6}]/\textsuperscript{4+} film by suppressing the response.

At potentials between +200 mV to +700 mV, the sensitivity of the response was low. A possible reason for the biosensor instability and poor response may be due to over-oxidation of the polymer at the higher positive potentials\textsuperscript{[104]}. It has also been reported\textsuperscript{[105]} that at these potentials there may be a significant decrease in conductivity of the polymer film and hence, a dramatic decrease in the sensitivity of the biosensor, which is associated with the over-oxidation of the polymer.
Figure 2.3:

Influence of applied potential on the sensitivity of the cholesterol response obtained with the PPy-COD-[Fe(CN)$_6$]$^{4-}$ biosensor. Polymerisation conditions: 0.5 mA/cm$^2$ for 120 s, 0.2 M pyrrole, 31.25 units/mL of COD and 5 mM [K$_4$Fe(CN)$_6$]. Amperometric measurement was made in 0.05 M phosphate buffer (pH 7.0) with a 5 mM cholesterol standard solution.
2.3.3 Effect of Polymerisation Time ($t_p$) and Current Density

The effect of polymerisation time on the amperometric response of the PPy-COD-[Fe(CN)$_6$]$^{4-}$ electrode is illustrated in Figure 2.4. The optimum cholesterol response was obtained with a polymerisation period of 100 s, indicating that at this time adequate coverage of the platinum electrode was achieved. Figure 2.4 also shows that the cholesterol response decreased with increasing polymerisation time beyond a period of 100 s. This may be due to the increased thickness of the PPy-COD-[Fe(CN)$_6$]$^{4-}$ film, which limits the rate at which the catalytic product can be detected at the platinum electrode, due to the increased diffusion barrier. Also, at longer polymerisation times the cholesterol response was not very reproducible and had a noisy baseline. A polymerisation time of 100 s was therefore used for all subsequent work. At values less than 100 s, the amperometric response obtained was much less sensitive and not reproducible. Possible explanations are that the time is not sufficient for an adequate amount of the COD to be incorporated into the PPy film, or that the time was insufficient for adequate coverage of the electrode by the PPy-COD-[Fe(CN)$_6$]$^{4-}$ film.

Figure 2.5 illustrates the influence of the applied current density, during the film formation, on the cholesterol response. An applied current density of 0.5 mA/cm$^2$ was chosen as the optimum for obtaining the most sensitive response for cholesterol. It is possible that at lower current densities the rate of polymerisation was slow and that the amount of enzyme incorporated into the film was lower.
Figure 2.4:

Influence of polymerisation time on the sensitivity of the cholesterol response obtained with PPy-COD-[Fe(CN)₆]⁴⁻ biosensor. Polymerisation conditions: 0.5 mA/cm², 0.2 M pyrrole, 31.25 units/mL of COD, 5 mM [K₄Fe(CN)₆]. Amperometric measurement was made in 0.05 M phosphate buffer (pH 7.0) at an applied potential of -200 mV using a 5 mM cholesterol standard solution.
Figure 2.5:

Influence of current density on the sensitivity of the cholesterol response obtained with PPy-COD-[Fe(CN)$_6$]$^{4-}$ biosensor. Polymerisation conditions: 0.2 M pyrrole, 31.25 units/mL of COD, 5 mM [K$_4$Fe(CN)$_6$] for 100 s. Amperometric measurement was made in 0.05 M phosphate buffer (pH 7.0) at an applied potential of -200 mV using a 5 mM cholesterol standard solution.
The decrease in the cholesterol response may also be due to the suppression in the diffusion of cholesterol in PPy-COD-[Fe(CN)$_6$]$^{4-}$ films because films formed at low current densities are usually very tight. This is in agreement with previous work on a similar PPy-COD-[Fe(CN)$_6$]$^{4-}$ electrode by Kajiya et. al. [225], which also shows a decrease in the response for films formed at similar low current densities. Current densities greater than 0.5 mA/cm$^2$ gave responses that were steadily decreasing. The PPy-COD-[Fe(CN)$_6$]$^{4-}$ films also appeared different, visually. The films became darker in colour with the increasing current density and appeared thicker. It has been previously reported [104] that there is a link between current density and film thickness. Generally, the higher the current density the thicker the polymer film, and the less sensitive the response. The thicker films tend to have an increased diffusion barrier between the platinum electrode and the PPy film and hence, decrease the response of the biosensor. The application of a current density of 0.5 mA/cm$^2$ resulted in good coverage of the platinum electrode with PPy-COD-[Fe(CN)$_6$]$^{4-}$ film. This permitted optimum interaction between the immobilised enzyme and the substrate, cholesterol.

2.3.4 Effect of Pyrrole and COD Concentration

Figure 2.6 shows the influence of pyrrole concentration on the amperometric response for cholesterol. The optimum response was obtained when the film was formed in 0.3 M pyrrole.
Figure 2.6:

Influence of pyrrole concentration on the sensitivity of the cholesterol response obtained with PPy-COD-[Fe(CN)_6]^{4-} biosensor. Polymerisation conditions: 0.5 mA/cm² for 100 seconds, 31.25 units/mL of COD and 5 mM [K₄Fe(CN)_6]. Amperometric measurement was made in 0.05 M phosphate buffer (pH 7.0) at an applied potential of -200 mV using a 5 mM cholesterol standard solution.
At lower concentrations, the sensitivity of the response was low, possibly due to inadequate coverage of the electrode and insufficient entrapment of COD. Beyond 0.3 M pyrrole the response decreased considerably and had a noisy background. The film appeared darker and thicker as well. This could be due to an increase in the thickness of the PPy-COD-[Fe(CN)₆]⁴⁻ film at these higher pyrrole concentrations. The decrease in the sensitivity could be also be due to an increase in the diffusion barrier caused by the thicker film.

The concentration of the enzyme in the polymerisation solution and therefore the amount incorporated into the PPy-COD-[Fe(CN)₆]⁴⁻ affects the response of the biosensor. In general, the higher the concentration of COD in the solution, the higher the amount of enzyme incorporated into the polymer film.

Figure 2.7 illustrates that the optimum response for cholesterol was obtained when 25 units/mL of COD was present in the monomer solution. The responses obtained with films formed at this enzyme concentration were reproducible and the baselines were smooth. At much higher concentrations, the sensitivity of the electrode seems to plateau. It appears that regardless of the amount of COD in the monomer solution beyond this concentration the response does not increase as expected. This suggests that a maximum amount of COD is incorporated into the film and no further incorporation is possible.
Figure 2.7:

Influence of COD concentration on the sensitivity of the cholesterol response obtained with PPy-COD-[Fe(CN)$_6$]$^{4-}$ biosensor. Polymerisation conditions: 0.5 mA/cm$^2$ for 100 seconds, 0.3 M pyrrole and 5 mM [K$_4$Fe(CN)$_6$]. Amperometric measurement was made in 0.05 M phosphate buffer (pH 7.0) at an applied potential of -200 mV using a 5 mM cholesterol standard solution.
2.3.5 Effect of Potassium Ferrocyanide Concentration

The addition of \([K_4Fe(CN)_6]\) to the polymerisation solution greatly enhanced the response and sensitivity of the biosensor. \([K_4Fe(CN)_6]\) is added as a mediator to promote electron transfer. Figure 2.8 illustrates that the sensitivity of the cholesterol response is very low in the absence of \([K_4Fe(CN)_6]\). It can be seen that the response increased by a factor of \(\sim 3\) after the addition of just 1 mM \([K_4Fe(CN)_6]\). The optimum \([K_4Fe(CN)_6]\) concentration chosen for this work was 5 mM. At this concentration, the mediator shuttles the electrons most efficiently, between the electrode and the COD in the PPy-COD-[Fe(CN)_6]^{4-} film. It is possible that at low concentrations the mediator is not mobile enough to act as an electron shuttle \[^{33}\]. At higher concentrations the cholesterol response decreased steadily, possibly due to the reduction of enzyme concentration in the presence of higher concentration of the mediator \[^{33}\]. At high mediator concentrations, there may be excessive diffusion within the film. Often excessive diffusion may result in mediator loss from the polymer to the bulk of the solution \[^{231}\]. This has a negative effect on the sensitivity and stability of the biosensor.

2.3.6 Comparison of Amperometric and Potentiometric Detection

The optimisation of various solution and instrumental parameters that influence the sensitivity of the PPy-COD-[Fe(CN)_6]^{4-} biosensor has been discussed. The optimum conditions of 0.3 M pyrrole, with 25 units/mL COD and 5 mM \([K_4Fe(CN)_6]\) in the film formation solution, for a
Figure 2.8:

Influence of $[K_4Fe(CN)_6]$ concentration on the sensitivity of the cholesterol response obtained with PPy-COD-$[Fe(CN)_6]^{4-}$ biosensor. Polymerisation conditions: 0.5 mA/cm$^2$ for 100 seconds, 0.3 M pyrrole and 25 units/mL COD. Amperometric measurement was made in 0.05 M phosphate buffer (pH 7.0) at an applied potential of -200 mV using a 5 mM cholesterol standard solution.
polymerisation time of 100 s at a current density of 0.5 mA/cm² were chosen based on both reproducibility and the sensitivity of the response under those conditions. The choice of the most sensitive detection method is critical for the development of a biosensor that is accurate, reproducible and sensitive. For this reason the use of amperometric and potentiometric detection modes were compared to identify the more sensitive and reliable detection mode. Factors used for comparison of the two detection modes are discussed below.

(a) Amperometric Detection

Figure 2.9 shows that when used in amperometric mode, the cholesterol biosensor appears to have two linear concentration ranges. The first achievable linear concentration range for cholesterol with the PPy-COD-\([\text{Fe(CN)}_6]^{4-}\) biosensor was between 49.5 and 198 \(\mu\text{M}\) cholesterol. The second linear range appeared to be between 297 and 495 \(\mu\text{M}\). The first linear range with a slope of 0.0002 mA/(cm²\(\mu\text{M}\)) appeared to be more sensitive than the second linear range with a slope of 3x10⁻⁵ mA/(cm²\(\mu\text{M}\)). It was not possible to measure cholesterol at concentrations between 0 and 49.5 \(\mu\text{M}\) because no response was obtained.

Figure 2.10 shows a typical amperometric responses for the PPy-COD-\([\text{Fe(CN)}_6]^{4-}\) biosensor. The experiments were performed by adding sequential aliquots to bring the total concentrations to 49.5, 99, 148.5, 247.5 \(\mu\text{M}\), respectively. The diagram shows that the baseline for the
Figure 2.9:

Calibration curves obtained for the PPy-COD-[Fe(CN)$_6$]$^{4+}$ biosensor by amperometric detection. Inlaid graphs show that the sensor displays 2 linear ranges: Linear Range 1 from [49.5 - 198] μM and Linear Range 2 from [297 - 495] μM cholesterol. Polymerisation conditions: 0.5 mA/cm$^2$ for 100 seconds, 0.3 M pyrrole 5 mM, [K$_4$Fe(CN)$_6$], and 25 units/mL COD. Amperometric measurement was made in 0.05 M phosphate buffer (pH 7.0) at an applied potential of -200 mV. (Key: Δ refers to the change in response.)
Figure 2.10:

Typical amperometric responses of PPy-COD-[Fe(CN)$_6$]$^{4-}$ biosensor to cholesterol. Cholesterol concentrations: (a) 49.5, (b) 99, (c) 148.5, (d) 198, (e) 247.5 μM. Polymerisation conditions: 0.5 mA/cm$^2$ for 100 seconds, 0.3 M pyrrole 5 mM, [K$_4$Fe(CN)$_6$] and 25 units/mL COD. Amperometric measurement was made in 0.05 M phosphate buffer (pH 7.0) and a measurement potential of -200 mV.
response is not very smooth and that the increase in the response was not as distinct as for the potentiometric response (Fig. 2.9). The minimum detectable amount of cholesterol with the amperometric detection was 49.5 µM.

(b) Potentiometric Detection

Figure 2.11 shows that the cholesterol biosensor also appears to have two linear concentration ranges. The first achievable linear concentration range for cholesterol with the PPy based-COD biosensor was between 0 and 247.5 µM cholesterol. The second linear range appeared to be between 247.5 and 500 µM. The first linear range with a slope of 0.0661 appeared to be much more sensitive than the second linear range with a slope of 0.0285. However, the R² value for the latter indicated that it had a higher linearity than the former. Figure 2.12 shows a typical potentiometric response for the PPy-COD-[Fe(CN)₆]⁴⁻ biosensor. The diagram shows that the baseline for the response is smooth and that the change in the response, is distinct when compared with the amperometric response (Fig. 2.8). The minimum detectable amount of cholesterol with the PPy-COD-[Fe(CN)₆]⁴⁻ electrode by potentiometric detection was 12.4 µM.

Table 2.1 clearly illustrates that when comparing the two detection modes, potentiometric detection of cholesterol with the PPy-COD-[Fe(CN)₆]⁴⁻ electrode, was the more sensitive method. The minimum detectable amount of 12.4 µM for potentiometric detection is four times lower than 49.5 µM obtained for amperometric detection.
Figure 2.11:

Calibration curves obtained for the PPy-COD-[Fe(CN)$_6$]$_{4-}$ biosensor by potentiometric detection. Inlaid graphs show that the sensor displays two linear ranges: linear range 1 from [12.4 - 247.5] $\mu$M and linear range 2 from [247.5 - 500] $\mu$M cholesterol. Polymerisation conditions: 0.5 mA/cm$^2$ for 100 seconds, 0.3 M pyrrole 5 mM, [K$_4$Fe(CN)$_6$] and 25 units/mL COD. Potentiometric measurement was made in 0.05 M phosphate buffer (pH 7.0).
Figure 2.12:

Typical potentiometric responses of PPy-COD-[Fe(CN)$_6$]$^{4-}$ biosensor to cholesterol. Cholesterol concentrations: (a) 49.5, (b) 99, (c) 148.5, (d) 198, (e) 247.5 μM. Polymerisation conditions: 0.5 mA/cm$^2$ for 100 seconds, 0.3 M pyrrole 5 mM, [K$_4$Fe(CN)$_6$] and 25 units/mL COD. Potentiometric measurement was made in 0.05 M phosphate buffer (pH 7.0).
Table 2.1:

Comparison of amperometric and potentiometric detection on the sensitivity of the cholesterol response obtained with PPy-COD-
\([\text{Fe(CN)}_6]^{4-}\) biosensor

<table>
<thead>
<tr>
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<th>Potentiometry</th>
<th>Amperometry</th>
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<tr>
<td>Type of Electrode System</td>
<td>2-electrode</td>
<td>3-electrode</td>
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<tr>
<td>Minimum Detectable Amount</td>
<td>12.4 $\mu$M</td>
<td>49.5$\mu$M</td>
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<td>(µM)</td>
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<td>Number of Linear Ranges</td>
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<tr>
<td>Linear Range 1</td>
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<tr>
<td>Sensitivity</td>
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<tr>
<td>$R^2$</td>
<td>12.4 – 247.5 $\mu$M</td>
<td>49.5 – 198 $\mu$M</td>
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<tr>
<td></td>
<td>0.066 mV/$\mu$M</td>
<td>0.0002 mA/(cm$^2$$\mu$M)</td>
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<td></td>
<td>0.970</td>
<td>0.950</td>
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<tr>
<td>Linear Range 2</td>
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<tr>
<td>Sensitivity</td>
<td></td>
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<tr>
<td>$R^2$</td>
<td>247.5 – 1000 $\mu$M</td>
<td>297 – 495 $\mu$M</td>
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<tr>
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<td>0.029 mV/$\mu$M</td>
<td>3x10$^{-5}$ mA/(cm$^2$$\mu$M)</td>
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<td>0.995</td>
<td>0.975</td>
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In addition, it is not possible to achieve a linear range below 49.5 µM for amperometric detection, while potentiometric detection has a sensitive achievable linear range at both very low concentrations of cholesterol and higher concentrations of cholesterol. Hence, sensitive quantification of cholesterol can therefore be accomplished by employing the potentiometric mode of detection. Table 2.2 provides a summary of the results obtained with the PPy-COD-[Fe(CN)$_6$]$^{4-}$ biosensor.

2.4 CONCLUSION

The fabrication of a cholesterol biosensor based on the incorporation of COD and a mediator into a PPy film for detecting cholesterol has been successfully demonstrated. The optimum conditions for formation of the PPy-COD-[Fe(CN)$_6$]$^{4-}$ electrode include 0.3 M pyrrole, 25 units/mL, 5 mM [K$_4$Fe(CN)$_6$], a polymerisation time of 100 s and an applied current density of 0.5 mA/cm$^2$. The optimum applied potential for the amperometric biosensing of cholesterol was −200 mV vs Ag/AgCl (3 M KCl). A comparison of amperometric and potentiometric modes of detection revealed that potentiometric detection was more sensitive and enabled detection of a wider concentration range, as demonstrated in Table 2.1. Although this section has illustrated that COD can be entrapped in a PPy film, the responses obtained were not as sensitive as expected. This may be due to the enzyme being only slightly doped into the polymer $^{[36]}$. Hence, it is necessary to consider alternate methods of immobilisation in order to develop a biosensor that is more sensitive.
2.5 SUMMARY OF RESULTS

Table 2.2:

Results and optimum conditions obtained using amperometric and potentiometric detection on the sensitivity of the cholesterol response obtained with PPy-COD-[Fe(CN)$_6$]$^{4-}$ biosensor

<table>
<thead>
<tr>
<th>OPTIMUM CONDITION / RESULT</th>
<th>Potentiometric Detection</th>
<th>Amperometric Detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Pyrrole]</td>
<td>0.3 M</td>
<td>0.3 M</td>
</tr>
<tr>
<td>[K$_4$Fe(CN)$_6$]</td>
<td>5 mM</td>
<td>5 mM</td>
</tr>
<tr>
<td>[COD]</td>
<td>25 units/mL</td>
<td>25 units/mL</td>
</tr>
</tbody>
</table>

**Film Formation Conditions**
- Polymerisation Time: 100 s
- Current Density: 0.5 mA/cm$^2$

**Measurement Conditions**
- Applied Potential: N/A, -200 mV

- Minimum Detectable Amount [Cholesterol]:
  - 12.4 M
  - 49.5 M

**Linear Range 1 [Cholesterol]**
- Sensitivity: 0.066 mV/μM, 0.2 A/(cm$^2$μM)
  - Linear Range: 12.4 – 247.5 μM

**Linear Range 2 [Cholesterol]**
- Sensitivity: 0.029 mV/μM, 0.03 A/(cm$^2$μM)
  - Linear Range: 247.5 – 500 μM
CHAPTER 3

FABRICATION OF A
BOVINE SERUM ALBUMIN–
GLUTARALDEHYDE–
CHOLESTEROL OXIDASE
BIOSENSOR FOR THE
MEASUREMENT OF FREE
CHOLESTEROL
CHAPTER 3

FABRICATION OF A BOVINE SERUM ALBUMIN-GLUTARALDEHYDE-CHOLESTEROL OXIDASE BIOSENSOR FOR FREE CHOLESTEROL

3.1 GENERAL INTRODUCTION

As discussed in the previous chapter the determination of cholesterol is very important in clinical diagnosis [1] since elevated levels have been strongly linked with several severe diseases including coronary heart disease, strokes and arteriosclerosis [2-7]. The use and development of biosensors for the determination of cholesterol has received considerable attention over the last decade. The development of PPy-based cholesterol biosensors has been popular since the pioneering research by Trettnak, Lionti and Mascini [3].

Although it has been demonstrated in Chapter 2 that COD can be entrapped in a PPy film, the resulting amperometric response obtained was inadequately sensitive. Similar low sensitivity has been reported in other work for PPy-based biosensors [225]. For this reason research interests in cholesterol biosensors has tended to consider alternate methods of immobilisation [185,191,192]. The range of immobilisation methods that have been considered include adsorption [232], covalent bonding [26], entrapment [3] and cross-linking [36]. Immobilisation methods having a high activity yield are desirable for
biosensors for economic reasons and for achievement of high sensitivity and functional stability. Among these, the use of cross-linking appears attractive due to the simplicity of the direct immobilisation of enzymes onto an electrode. Chemical cross-linking is amongst the more commonly used methods for immobilising enzymes onto electrode surfaces. In addition to the advantages listed previously, the method is rapid, simple to perform and has a very wide range of applicability. Hence, this immobilisation method was chosen as an alternate method of enzyme immobilisation with the view to develop a more robust biosensor for the determination of cholesterol.

In principle the procedure is simple: a drop of solution containing the enzyme to be immobilised is combined with BSA which is a lysine-rich auxiliary protein also known as a spacer and GLA which is a cross-linking or bifunctional reagent. The GLA links the protein molecules together and forms an insoluble gel-matrix, in which the enzyme is immobilised. There are several other bifunctional reagents that can be used, but GLA appears to be the most satisfactory. Dong et al. fabricated a biosensor where COD was cross-linked with GLA onto a Pd dispersed electrode, and a layer of o-PPD was electropolymerised on the COD-GLA. However, to our knowledge there has not been any previously reported work, which utilised a chemical cross-linking method for formation of BSA-GLA-COD film on a platinum electrode for the development of a cholesterol biosensor. However, this method was attractive for the immobilisation of COD and the development of cholesterol biosensors, during this study, for all of the advantages listed above.
The aim of the research described in this chapter is to develop a cholesterol biosensor for the measurement of free cholesterol, by chemical cross-linking of COD with BSA-GLA. The optimisation of the components of the sensing layer, as well as the influence of drying time and storage conditions, will be described. The sensitivity and response of the sensor particularly with regards to the minimum detectable amount and the determination of the linear range will also be discussed.

3.2 EXPERIMENTAL

Laboratory conditions, instrumentation, glassware, procedures for electrode preparation and cholesterol standards were as or prepared as described in 2.2 of Chapter 2, unless otherwise stated.

3.2.1 Chemicals and Standard Solutions

The glutaraldehyde (25 % v/v aqueous solution), bovine serum albumin, uric acid and ascorbic acid were purchased from the Sigma-Aldrich Chemical Company. Stock solutions of 15% w/v BSA and 10% v/v GLA were prepared and stored in the refrigerator at ~ 4°C. These were diluted later to give the appropriate concentrations. The GLA stock solution was deliberately lower than the BSA stock solution in the mixture for the layers, to avoid excess cross-linking and hence denaturing [184] of the COD and/or CE.

A 10 mM stock solution of ascorbic acid was prepared by dissolving an appropriate amount (0.04403 g) of the acid with Milli-Q water and the volume was adjusted to 25.00 mL. A 10 mM stock solution of uric acid
was prepared by dissolving an appropriate amount of the acid (0.04202 g) in an equimolar amount of Li₂CO₃ (0.0185 g) with Milli-Q water and the volume was adjusted to 25.00 mL.

### 3.2.2 Procedures

(a) *Enzyme Immobilisation*

The immobilisation of the COD was a two step procedure. To improve adhesion of the BSA-GLA-COD layer it was found necessary to have an initial thin layer of BSA-GLA. A 1 μL volume of the mixture as described in Table 3.1 for layer 1 was initially spread onto the platinum electrode and allowed to air dry until the mixture had gelatinised and hardened. Then 3 μL of the second mixture for layer 2 was spread on top of the first layer. This layer was allowed to air dry until the film had hardened and gelatinised. The electrode was washed under a stream of Milli-Q water to remove any loosely bound molecules, prior to analysis.

**Table 3.1:**

Composition of each layer for BSA-GLA-COD electrode

<table>
<thead>
<tr>
<th>Mixture for Layer</th>
<th>Vol. BSA (μL)</th>
<th>Vol. GLA (μL)</th>
<th>Vol. COD (μL)</th>
<th>Vol. of Mixture Used (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5 (12.5% w/v)</td>
<td>1 (1.7% v/v)</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>5* (6.8% w/v)</td>
<td>5* (4.5% v/v)</td>
<td>1* (23 units/mL)</td>
<td>3</td>
</tr>
</tbody>
</table>

*Denotes amounts that were varied during optimisation of parameters*
(b) **Potentiometric Measurement**

A two-electrode electrochemical cell was employed for all measurements. The cell contained 20 mL of phosphate buffer (pH 7.0) which was magnetically stirred. The response of the sensor was measured potentiometrically by additions of the standard cholesterol solution. The response towards ascorbic acid and uric acid was tested in the same way by additions of the stock solutions into the measurement cell prior to the potentiometric measurement. In both cases, the potential was allowed to stabilise completely, which varied between 20 and 30 minutes, before any additions were made.

The minimum detectable amount was determined by continuous additions of cholesterol until a response was obtained. The experiment was repeated three times to confirm the minimum detectable amount obtained.

(c) **Optimisation of [COD], [BSA] and [GLA]**

To determine the optimum concentration to be used in the formation of the polymeric film, the volume of components in the mixture used for the layer 2 was varied. The volumes of the other two components were held constant as illustrated in Tables 3.2-3.4. Reproducibility was determined in terms of mean deviation (MD). The sample number analysed was 3 (n = 3)
(d) **Film Drying**

Each layer was allowed to air-dry in the laboratory under the conditions listed previously. For accelerated drying a hand held Braun Silencio hair-drier, mounted above the film on a retort stand, was used to dry layer 2.

(e) **Storage Study**

The electrode prepared for the wet storage study was stored, in the refrigerator, after each daily analysis, in a polyethylene tube containing fresh 0.05 M phosphate buffer (pH 7.0).

The electrode prepared for the dry storage study was stored in the refrigerator, after each daily analysis. It was suspended, by an o-ring, in a dry and empty polyethylene tube, to avoid damage to the film.

(f) **Pre-Treatment and Analysis of Serum Samples**

The blood serum samples (which are usually discarded after analysis) were obtained from The Royal Prince Alfred Hospital. The samples at the hospital were pre-treated and analysed by an enzymatic colorimetric method [5,19,30]. This involves the addition of a cholesterol reagent at the start of the reaction. Cholesterol is determined enzymatically using CE to yield free cholesterol and fatty acids, and COD which converts cholesterol to a ketone and hydrogen peroxide. The hydrogen peroxide produced forms a red dyestuff by reacting with 4-aminophenazone and phenol under the catalytic action of peroxidase, which is also present. The colour
intensity is directly proportional to the concentration of cholesterol and can be determined photometrically.

The cholesterol in the serum samples was determined using the biosensor, by employing a standard additions approach. After the background potential for the blank buffer solution had stabilised, the required dilution of serum was established by adding aliquots of serum to the cell until a reasonably sensitive response was obtained. A dilution of 1:200 was found to be adequate.

As the samples were pre-treated at the hospital most of the esterified cholesterol in the serum was in the form of free cholesterol.

Great care was taken while working with the blood serum samples to prevent spillage and contact. Gloves were worn at all times to minimise the risk of contact with the serum. After conducting experiments with the blood serum, hands were scrubbed for a minimum of 2 minutes with a hospital grade disinfectant, followed by rinsing with water.

3.3 RESULTS AND DISCUSSION

3.3.1 Optimisation of Components in the Sensing Layer

The ratio of membrane components is critical for the development of a biosensor that is sensitive and reliable. Hence, the concentrations and/or ratios of the components that are combined to form the sensing layer need to be optimised. For this purpose the range of factors considered are discussed below.
Film characterisation by cyclic voltammetry was necessary in Chapter 2, to ascertain whether the COD was incorporated into the PPy film from the monomer solution during electropolymerisation. However, characterisation by cyclic voltammetry is not necessary in this section, because the COD is physically mixed into the polymer matrix, before it is spread onto the electrode, hence its presence in the film is certain.

(a) Optimisation of COD Concentration

The results obtained for varying the [COD] in the outer layer are illustrated in Figure 3.1 and tabulated in Table 3.2. Both show that the optimum response (0.152 ± 0.006 mV/μM) was obtained when 23 units/mL was incorporated into the polymeric matrix. The responses obtained with films formed at this [COD] were reproducible (MD ± 3.60 %, n = 3) and the baselines were fairly smooth. At lower concentrations, the response was not as sensitive. This is possibly due to lower enzyme activity, resulting from an insufficient enzyme reserve in the matrix.

At higher concentrations, the response appears to decrease and the sensitivity seems to plateau. This is thought to be due to ineffective mass transfer. The highest sensitivities are achieved when there is large enzyme activity within a thin enzyme layer and an effective external mass transfer is provided [40]. Interestingly the achievable linear concentration range for cholesterol was not influenced significantly by variation of [COD] in the outer layer, as illustrated in Table 3.2.
Figure 3.1:

Influence of varying [COD] on the sensitivity of the cholesterol response obtained with the BSA-GLA-COD biosensor. Concentrations of components in sensing layer were as described in Table 3.1, except that [COD] was varied. Potentiometric measurement was made in 0.05 M phosphate buffer (pH 7.0) using a 5 mM cholesterol standard solution.
Table 3.2:

Influence of varying [COD] on the sensitivity of the cholesterol response obtained with the BSA-GLA-COD biosensor

<table>
<thead>
<tr>
<th>Film Composition in (µL) Volumes BSA:GLA:COD</th>
<th>Sensitivity (mV/µM)</th>
<th>$R^2$</th>
<th>Linear Range [cholesterol] (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 : 5 : $\frac{1}{2}$</td>
<td>0.103</td>
<td>0.933</td>
<td>49.5 - 396</td>
</tr>
<tr>
<td>5 : 5 : 1</td>
<td>0.152</td>
<td>0.950</td>
<td>49.5 - 396</td>
</tr>
<tr>
<td>5 : 5 : 2</td>
<td>0.112</td>
<td>0.955</td>
<td>49.5 – 396</td>
</tr>
<tr>
<td>5 : 5 : 4</td>
<td>0.107</td>
<td>0.947</td>
<td>99 – 396</td>
</tr>
<tr>
<td>5 : 5 : 8</td>
<td>0.107</td>
<td>0.959</td>
<td>99 - 396</td>
</tr>
</tbody>
</table>

**Corresponding [Concentrations] of Film Composition:**

[COD] (units/mL) : $\frac{1}{2}$ µL = 12, 1 µL = 23, 4 µL = 71, 8 µL = 111

[BSA] : 5 µL of BSA = 6.8% w/v

[GLA] : 5 µL of GLA = 4.5% v/v
(b) Optimisation of BSA Concentration

BSA is a functionally inert, lysine-rich auxiliary protein and has several important functions in chemical cross-linking. It can increase the total protein concentration, allowing gel formation from solutions that would otherwise give only soluble oligomers \(^{207}\). BSA also decreases the porosity of the film thereby making it more stable, thus increasing the responsiveness of the film \(^{234}\). The results obtained for varying the [BSA] are illustrated in Figure 3.2 and presented in Table 3.3. Both show that the most sensitive response (0.152 ± 0.006 mV/µM) was obtained when 6.8% w/v was used as a component of the mixture used for the outer layer. The responses obtained with films formed at this BSA concentration were reproducible (MD ± 0.67 %, n = 3) and the baselines were fairly smooth. Table 3.3 shows that sensitivity of the cholesterol response was low at BSA concentrations of 1.2 – 3.8% w/v and at concentrations of 5 – 6% w/v the response was not as sensitive as in presence of 6.8% w/v. A possible explanation is that there was excess cross-linking of the active enzyme due to an insufficient amount of BSA protein molecules. BSA behaves as a spacer and, hence, minimises the close proximity problems, which can be caused by cross-linking a single enzyme \(^{46,184}\). At concentrations higher than 6.8% w/v the response was not as sensitive and tended to plateau, as illustrated in Figure 3.2. At these high BSA levels, the film does not have good mechanical properties. The protein content may also be too high, resulting in the enzyme molecules being spaced too far apart resulting in a decrease in COD activity. The COD activity within the enzyme layer is not large enough
Figure 3.2:

Influence of varying [BSA] on the sensitivity of the cholesterol response obtained with the BSA-GLA-COD film. Concentrations of components in sensing layer were as described in Table 3.1 except that [BSA] was varied. Potentiometric measurement was made in 0.05 M phosphate buffer (pH 7.0) using a 5 mM cholesterol standard solution.
Table 3.3:

Influence of varying [BSA] on the sensitivity of the cholesterol response obtained with the BSA-GLA-COD biosensor

<table>
<thead>
<tr>
<th>Film Composition in (μL) Volumes BSA:GLA:COD</th>
<th>Sensitivity (mV/μM)</th>
<th>R²</th>
<th>Linear Range [cholesterol] (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>½ : 5 : 1</td>
<td>No Response</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>1 : 5 : 1</td>
<td>No Response</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>2 : 5 : 1</td>
<td>No Response</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>3 : 5 : 1</td>
<td>0.097</td>
<td>0.975</td>
<td>99 - 396</td>
</tr>
<tr>
<td>4 : 5 : 1</td>
<td>0.126</td>
<td>0.960</td>
<td>99 – 396</td>
</tr>
<tr>
<td>5 : 5 : 1</td>
<td>0.152</td>
<td>0.973</td>
<td>99 – 396</td>
</tr>
<tr>
<td>6 : 5 : 1</td>
<td>0.101</td>
<td>0.950</td>
<td>99 – 396</td>
</tr>
<tr>
<td>8 : 5 : 1</td>
<td>0.095</td>
<td>0.944</td>
<td>99 – 396</td>
</tr>
</tbody>
</table>

Corresponding [Concentrations] of Film Composition:

[BSA] (% w/v) : ½ μL = 1.2, 1 μL = 2.1, 2 μL = 3.8, 3 μL = 5, 4 μL = 6, 5 μL = 6.8, 6 μL = 7.5, 8 μL = 8.6

[GLA] : 5 μL of GLA = 4.5% v/v

[COD] : 1 μL of COD = 23 units/mL

94
and an effective external mass transfer is not provided, high sensitivities are not achieved [235].

(c) Optimisation of GLA Concentration

GLA is a cross-linking reagent, which links the protein molecules together and forms an insoluble gel-matrix, in which the COD is immobilised [31,184]. The GLA stock solution of 10% v/v, in the polymeric mixture for the sensing layers, was deliberately lower in concentration than the BSA stock solution of 15% w/v. This was done to avoid excess cross-linking and, hence, denaturing [184] of the enzyme.

The results obtained for varying the GLA are illustrated in Figure 3.3 and tabulated in Table 3.4. Both show that the most sensitive response (0.137 ± 2 x 10^-4 mV/μM) was obtained when 4.5% v/v was used to form the enzyme-immobilisation matrix. The responses obtained with films formed at this GLA concentration were reproducible (MD ± 0.14 %, n = 3) and the baselines were fairly smooth. At concentrations between 0.8 and 4% v/v GLA, the response was not as sensitive. The films produced also took longer to dry and the colour was opaque light yellow as opposed to the films for 4.5% v/v GLA that were a dark sienna-yellow colour after drying.

It is possible that lower concentrations of GLA were not sufficient to allow adequate cross-linking of the COD. As mentioned previously the sensitivity and stability of the biosensor remains constant as long as an enzyme reserve is present. If there is an inadequate amount of immobilised COD then it is possible to assume that a good reaction rate will not be achieved, hence resulting in a decrease in the sensitivity [235].

95
Figure 3.3:

Influence of varying [GLA] on the sensitivity of the cholesterol response obtained with BSA-GLA-COD biosensor. Concentrations of components in sensing layer were as described in Table 3.1 except that [GLA] was varied. Potentiometric measurement was made in 0.05 M phosphate buffer (pH 7.0) using a 5 mM cholesterol standard solution.
Table 3.4:

Influence of varying [GLA] on the sensitivity of the cholesterol response obtained with the BSA-GLA-COD biosensor.

<table>
<thead>
<tr>
<th>Film Composition in (μL) Volumes BSA:GLA:COD</th>
<th>Sensitivity (mV/μM)</th>
<th>R²</th>
<th>Linear Range [cholesterol] (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 : ½ : 1</td>
<td>0.089</td>
<td>0.970</td>
<td>148.5 - 396</td>
</tr>
<tr>
<td>5 : 1 : 1</td>
<td>0.097</td>
<td>0.958</td>
<td>99 - 396</td>
</tr>
<tr>
<td>5 : 3 : 1</td>
<td>0.086</td>
<td>0.977</td>
<td>99 - 396</td>
</tr>
<tr>
<td>5 : 4 : 1</td>
<td>0.088</td>
<td>0.958</td>
<td>99 - 396</td>
</tr>
<tr>
<td>5 : 5 : 1</td>
<td>0.137</td>
<td>0.973</td>
<td>99 - 396</td>
</tr>
<tr>
<td>5 : 6 : 1</td>
<td>0.08</td>
<td>0.983</td>
<td>49.5 - 396</td>
</tr>
<tr>
<td>5 : 8 : 1</td>
<td>0.081</td>
<td>0.951</td>
<td>99 - 396</td>
</tr>
</tbody>
</table>

Corresponding [Concentrations] of Film Composition:

[GLA] (% w/v) : ½ μL = 0.8, 1 μL = 1.4, 3 μL = 3.3, 4 μL = 4, 5 μL = 4.5, 6 μL = 5, 8 μL = 5.7

[BSA] : 5 μL of GLA = 6.8% v/v

[COD] : 1 μL of COD = 23 units/mL
At concentrations of 5 – 5.7% v/v GLA, the sensitivity decreased dramatically. A possible reason for the decrease in the sensitivity is enzyme activity losses due to chemical alterations of the catalytically essential sites of the protein \cite{46}. Enzymes tend to lose activity when cross-linked directly to GLA \cite{236}. The extent of the deleterious effect is dependent on the nature of the enzyme \cite{189,194-197}. It is possible in the case of COD that the response is sensitive reaching the optimum at 4.5% v/v GLA. The sensitivity then decreases with suppression in the responses due to loss of activity and substrate diffusion problems, caused by the increased concentration of GLA. The less sensitive responses observed for the higher GLA concentrations may be the result of an increase in the film thickness. The thicker the film, the larger the diffusion barrier. Hence, the amount of analyte reacting at the electrode surface is reduced which in turn reduces the sensitivity of the biosensor \cite{234,237,238}. This is due to the additional diffusion resistance \cite{234}. Good diffusion properties are important for free movement of substrate and product and a good rate of reaction with immobilised enzymes \cite{235}. The highest sensitivities are achieved when there is large enzyme activity within a thin enzyme layer and an effective external mass transfer is provided \cite{235}.

3.3.2 Optimisation of Drying Time

(a) Air Drying Time

Figure 3.4 shows that polymeric films formed by chemical cross-linking of COD, BSA and GLA that were air-dried for 30 minutes gave the most
Figure 3.4:

Influence of varying air-dry time in BSA-GLA-COD film on the sensitivity of the cholesterol response. Concentrations of components in sensing layer were as described in Table 3.1. Potentiometric measurement was made in 0.05 M phosphate buffer (pH 7.0).
sensitive response. At times below 30 minutes, no responses were obtained. At times less than 10 minutes the film was slightly watery in appearance and did not appear to have dried, probably due to insufficient time for the GLA to polymerise. Because of the incomplete cross-linking the film-solution mixture does not harden to form an insoluble gel matrix and the COD is not successfully immobilised. Hence, little, if any, reaction takes place at the biosensor surface and therefore the lack of response.

Table 3.5 and Figure 3.4 show that at times higher than 30 minutes the sensitivity was erratic: decreased, increased (but the sensitivity was well below the optimum) and decreased again. This could be due to the poor diffusion properties and lack of effective external mass transfer caused by excess cross-linking of the active COD [207,234,239]. Although this behaviour appears to have no real trend, it is characteristic of cross-linking with GLA [207]. The composition of GLA changes over time as it polymerises at room temperature, hence affecting the nature of the polymer matrix and the resulting sensitivity [207].

(b) **Accelerated Drying Time**

There are no results for this method of film drying because none of the films produced in this way gave any response. Even accelerated drying for a short time of 5 minutes, resulted in very noisy and unstable background readings. The additions of cholesterol to the buffer solution gave no response with these films, indicating that there was no reaction occurring
### Table 3.5:

Influence of varying air-dry time in BSA-GLA-COD film on the sensitivity of the cholesterol response

<table>
<thead>
<tr>
<th>Drying Time (minutes)</th>
<th>Sensitivity (mV/ M)</th>
<th>$R^2$</th>
<th>Linear Range [Cholesterol] (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt; 30</td>
<td>No Response</td>
<td>No Response</td>
<td>No Response</td>
</tr>
<tr>
<td>30</td>
<td>0.137</td>
<td>0.966</td>
<td>99 - 396</td>
</tr>
<tr>
<td>60</td>
<td>0.074</td>
<td>0.961</td>
<td>99 – 396</td>
</tr>
<tr>
<td>90</td>
<td>0.113</td>
<td>0.952</td>
<td>49.5 - 396</td>
</tr>
<tr>
<td>120</td>
<td>0.086</td>
<td>0.972</td>
<td>99 - 396</td>
</tr>
</tbody>
</table>
between the substrate and the immobilised COD. This behaviour is characteristic of enzyme inactivation \[^{[234]}\]. It is possible that the intense heat of the hair drier during the attempt to accelerate the drying thermally denatured the COD. Hence, the inactive COD will not catalyse the oxidation of cholesterol \[^{[235]}\].

3.3.3 Determination of Optimum Storage Conditions

The useful lifetime of biosensors usually depends on the retention of the enzyme activity within the sensing layer. This may vary from months to days depending on the method of manufacture, stability of the enzyme and most importantly, the storage conditions \[^{[240]}\].

(a) Wet Storage

A comparison of the BSA-GLA-COD biosensor responses under wet and dry storage conditions is illustrated in Figure 3.5. The sensor stored in the phosphate buffer showed a decline in the sensitivity over the 6-day period. The longer the biosensor was stored in the buffer, the less sensitive the response. This loss in sensitivity was probably due to a decrease in the [COD] in the outer layer of the biosensor \[^{[235]}\] caused by leaching of the COD from the polymeric matrix. The decrease in sensitivity can also be attributed to an increase in porosity of the film leading to poor mechanical properties of the film and poor film stability \[^{[31,184]}\].
(b) Dry Storage

Figure 3.5 also shows that the BSA-GLA-COD biosensor stored under dry conditions had a more stable sensitivity over the six-day period. As mentioned previously the enzyme loading determines the sensitivity of the biosensor to a major extent. As long as the COD reserve remains constant in the outer layer, the sensitivity of the biosensor will remain fairly constant \(^{46}\). Storing an electrode under dry conditions essentially maintains the mechanical properties and stability of the film. Leaching does not occur in a dry environment therefore, the enzyme content will remain fairly constant. Storage in the fridge also helps maintain the activity of the COD by preventing the thermal denaturing of the COD that can occur if the electrode is stored at room temperature \(^{235}\). For these reasons the sensitivity of the BSA-GLA-COD biosensor stored under dry conditions, remained essentially constant.

3.3.4 Interference Study

In complex media various substances which are electroactive may exist in the sample \(^{54}\). The specificity of a biosensor against these interferences is of primary importance because it reduces the need for pre-treatment, such as separation \(^{241}\) and makes the biosensor more amenable for automated analysis. There are a variety of interferents that may be present in biological samples. Thus, biosensors that are used should be specific enough to discriminate against these coexisting substances \(^{54,241,242}\). Alternatively other approaches that suppress the interferences may be considered.
Figure 3.5:

Comparison of storage conditions on the sensitivity of the cholesterol response obtained with BSA-GLA-COD biosensor. 0.05 M phosphate buffer (pH 7.0) was used for wet storage. Conditions were as described for Figure 3.4.
For the purposes of this study two of the interfering species frequently found in biological media [32], ascorbic acid and uric acid, are considered.

(a) Effect of Ascorbic Acid

Ascorbic acid is considered to be a major interferent to most biosensors including COD sensors [33]. This is due to a combination of its relatively high concentrations in biological samples and low oxidation potential. Figure 3.6 shows that the presence of ascorbic acid interferes with the response significantly. Table 3.6 shows that the addition of 0.001 mM ascorbic acid resulted in the suppression of the cholesterol response by 70.3%. The effect of ascorbic acid on the response is even more dramatic at higher concentrations. Table 3.6 shows that the presence of 0.1 mM ascorbic acid suppressed the response completely. The background was very noisy and the potential did not stabilise. This is due to the very high concentration of ascorbic acid in the solution that is oxidised together with the hydrogen peroxide and therefore does not allow the production of a sensitive response [54]. This suppressing effect of ascorbic acid is very similar to results obtained for other cholesterol biosensors [3,21,28] with different enzyme immobilisation method. Similar results were reported for cholesterol biosensor based on: electropolymerisation of pyrrole [3], COD immobilised on collagen [21,28] and total cholesterol determination on tubular carbon electrodes [40]. The reported concentrations of ascorbic acid in serum ranges from 45 – 90 μM [3]. This means that in whole serum the presence of ascorbic acid would affect the response of the sensor, and this point needs to be taken into consideration.
Figure 3.6:

Influence of ascorbic acid on the sensitivity of the cholesterol response obtained with BSA-GLA-COD biosensor. Concentrations of components in sensing layer were as described in Table 3.1. Potentiometric measurement was made in 0.05 M phosphate buffer (pH 7.0) using a 5 mM cholesterol standard solution.
Table 3.6:

Influence of [ascorbic acid] on the sensitivity of the cholesterol response obtained with BSA-GLA-COD biosensor

<table>
<thead>
<tr>
<th>[Ascorbic Acid] (mM)</th>
<th>Sensitivity (mV/ M)</th>
<th>Percentage of Response Suppressed (%)</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.3641</td>
<td>0</td>
<td>0.995</td>
</tr>
<tr>
<td>0.001</td>
<td>0.1081</td>
<td>70.3</td>
<td>0.987</td>
</tr>
<tr>
<td>0.01</td>
<td>0.0589</td>
<td>83.7</td>
<td>0.982</td>
</tr>
<tr>
<td>0.1</td>
<td>No Response</td>
<td>100</td>
<td>N/A</td>
</tr>
</tbody>
</table>
However, during the course of this study the analysis of serum was performed with a 1:200 dilution. This dilution would result in the presence of 0.23 – 0.45 μM ascorbic acid in the diluted serum sample. Hence, it is unlikely that these low concentrations of ascorbic acid will affect the performance of the cholesterol biosensor when used for analysis of serum samples. This high dilution minimises the matrix effects of the serum and the effects of the electroactive interferents present, such as ascorbic and uric acid.

(b) Effect of Uric Acid

Uric acid has also been classed as a common interferent, but not as limiting as ascorbic acid [4]. This is illustrated in Figure 3.7 and Table 3.7, which shows that in the presence of 0.01 mM uric acid, the sensitivity of the biosensor response was suppressed by 50%. This is consistent with recent work reported which has suggested that the presence of uric acid would degrade the sensitivity of the enzyme based biosensors [242].

Low concentrations of uric acid (0.001 mM) is ~23% less suppressing than the same concentration of ascorbic acid. The responses obtained for concentrations of uric acid less than 1 mM had smooth baselines and the responses were well defined. The reported serum levels of uric acid in serum ranges between 140 and 420 μM [3]. These levels are high, and based on the results obtained in Figure 3.7 and Table 3.7, the presence of uric acid in whole serum would suppress the response. However, as mentioned previously during the course of this study the analysis of
Figure 3.7:

Influence of uric acid on the sensitivity of the cholesterol response obtained with the BSA-GLA-COD. Concentrations of components in sensing layer were as described in Table 3.1. Potentiometric measurement was made in 0.05 M phosphate buffer using a 5 mM cholesterol standard solution.
Table 3.7:

Influence of [uric acid] on the sensitivity of the cholesterol response obtained with the BSA-GLA-COD

<table>
<thead>
<tr>
<th>[Uric Acid] (mM)</th>
<th>Sensitivity (mV/ M)</th>
<th>Percentage of Response Suppressed (%)</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.2942</td>
<td>0</td>
<td>0.995</td>
</tr>
<tr>
<td>0.001</td>
<td>0.1540</td>
<td>47.7</td>
<td>0.994</td>
</tr>
<tr>
<td>0.01</td>
<td>0.1472</td>
<td>50</td>
<td>0.915</td>
</tr>
<tr>
<td>0.1</td>
<td>0.0613</td>
<td>79.2</td>
<td>0.897</td>
</tr>
</tbody>
</table>
serum was performed with a 1:200 dilution. At this dilution, uric acid will be present in the serum sample within a range of 0.7 – 2.1 μM. It is unlikely that these low concentrations of uric acid will affect the performance of the BSA-GLA-COD biosensor for the determination of cholesterol in diluted serum samples.

### 3.3.5 Linear Concentration Range and Minimum Detectable Amount

The minimum detectable amount of cholesterol with the BSA-GLA-COD biosensor, fabricated by chemical cross-linking, was 2.5 μM. Evidently this method of electrode fabrication has produced a very sensitive biosensor. The BSA-GLA-COD cholesterol biosensor appears to have two linear concentration ranges. Figure 3.8 (a) shows that the first achievable linear concentration range for cholesterol with the cross-linked BSA-GLA-COD biosensor was between 2.5 and 25 μM cholesterol.

Figure 3.8 (b) shows that a second linear concentration range was obtained between 49.5 and 297 μM. The first linear range with a slope of 1.1115 mV/μM appeared to be more sensitive than the second linear range with a slope of 0.1738 mV/μM.

The presence of these linear ranges at such different cholesterol concentrations indicates that sensitive determination of cholesterol can be accomplished for both low and higher concentrations of cholesterol.
Figure 3.8 (a):

Calibration curves for cholesterol obtained with the BSA-GLA-COD biosensor. The inlaid "Linear Range 1" graph shows that the sensor displays a linear response in the range of [2.5 – 25] μM cholesterol. (Key: ΔE refers to the change in potential)
Figure 3.8 (b):

Calibration curve for cholesterol response obtained with the BSA-GLA-COD Biosensor. The graph shows that the sensor displays a linear response “Linear Range 2” in the range of [49.5 – 297] μM cholesterol.
The reproducibility of fabrication of an electrode is equally as important as the reproducibility of measurements with an individual electrode. For this reason the sensitivity of the BSA-GLA-COD biosensor over several different fabrications was analysed. The achieved sensitivity \((0.1515 \pm 1.3\) \% \) mV/\(\mu\)M indicates that the BSA-GLA-COD biosensor sensitivities given, were consistent with repeated use of the biosensor over several different fabrications.

3.3.6 Percentage Recovery and Application to Serum Samples

Table 3.8 shows that excellent recoveries, in the range of \((94.3 - 110.5)\) \% were achieved, with the BSA-GLA-COD biosensor for the quantification of known spiked amounts of cholesterol. These results demonstrate that accurate and sensitive measurement of cholesterol can be accomplished with the biosensor. The standard deviation values also demonstrate that the sensor allows for reproducible quantification. The higher recovery data obtained for 9.9 \(\mu\)M of cholesterol added, may be due to some systematic error, however, the results are quite reproducible. The same reasoning applies to 2.475 \(\mu\)M cholesterol added, where slightly lower recovery was obtained.

Figure 3.9 shows a typical potentiogram obtained for quantification of cholesterol in serum. Table 3.9 shows that the values obtained for the sample Ser/P1 5009-1 using the BSA-GLA-COD biosensor, had a much larger variation to the value obtained by the standard colorimetric method used at the Royal Prince Alfred Hospital. However, the results obtained
Table 3.8:
Sensitivity of BSA-GLA-COD biosensor in percentage recovery of free cholesterol

<table>
<thead>
<tr>
<th>[Cholesterol] Added (M)</th>
<th>[Cholesterol] Found (M)</th>
<th>Percentage Recovery (MD, n = 3) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.475</td>
<td>2.334</td>
<td>94.3 ± 1.9</td>
</tr>
<tr>
<td>4.95</td>
<td>4.95</td>
<td>100 ± 2.1</td>
</tr>
<tr>
<td>9.9</td>
<td>10.94</td>
<td>110.5 ± 3.6</td>
</tr>
</tbody>
</table>
Figure 3.9:

Typical potentiometric responses obtained for the quantification of cholesterol in serum with the BSA-GLA-COD biosensor. Cholesterol concentrations: (Std. 1) 6.2; (Std. 2) 12.4 and (Std. 3) 18.6 μM. Concentration of components in sensing layer were as described in Table 3.1.
Table 3.9:

Determination of cholesterol in serum samples with the BSA-GLA-COD biosensor

<table>
<thead>
<tr>
<th>Sample Number</th>
<th>[Cholesterol] measured by Clinical Method (mM)</th>
<th>[Cholesterol] measured by Biosensor (MD, n = 3) (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ser/P1 5006-4</td>
<td>4.09</td>
<td>4.55 ± 1.57</td>
</tr>
<tr>
<td>Ser/P1 5009-1</td>
<td>4.15</td>
<td>5.69 ± 0.1</td>
</tr>
<tr>
<td>Ser/P1 5006-2</td>
<td>4.04</td>
<td>4.29 ± 0.13</td>
</tr>
</tbody>
</table>
for the measurement of cholesterol in samples Ser/P1 5006-4 and Ser/P1 5006-2 using the BSA-GLA-COD biosensor, were reasonably closer to the values obtained by the standard colorimetric method, although the result for Ser/P1 5006-4 is not as reproducible as for the other samples, this may be due to a systematic error as previously mentioned.

The advantage of using the biosensor for serum analysis was that further sample pre-treatment was not necessary. The method using the biosensor is also rapid, unlike the time-consuming clinical method.

3.4 CONCLUSION

A BSA-GLA-COD biosensor has been successfully fabricated for accurate potentiometric measurement of free cholesterol. The fabrication of the biosensor by chemical cross-linking of COD to BSA and GLA takes only 30 minutes. The achievable minimum detectable amount of cholesterol with this biosensor is 2.5 μM and a linear concentration range of 2.5 – 25 μM was achieved in the most sensitive range. Recovery studies gave excellent results with percentage recoveries in the range (94.3 – 110.5%). The optimum conditions accomplished for this biosensor are summarised in Table 3.9.

The biosensor was successfully employed for the determination of cholesterol in blood serum.
3.5 SUMMARY OF RESULTS

Table 3.10:

Summary of results and optimum conditions obtained using the BSA-GLA-COD biosensor for free cholesterol measurement

<table>
<thead>
<tr>
<th>PARAMETER</th>
<th>ABBREVIATION</th>
<th>OPTIMUM CONDITION or RESULT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration of BSA</td>
<td>[BSA]</td>
<td>6.8% w/v</td>
</tr>
<tr>
<td>Concentration of GLA</td>
<td>[GLA]</td>
<td>4.5% v/v</td>
</tr>
<tr>
<td>Concentration of COD</td>
<td>[COD]</td>
<td>23 units/mL</td>
</tr>
<tr>
<td>Air-Dry Time</td>
<td>T</td>
<td>30 minutes</td>
</tr>
<tr>
<td>Accelerated-Drying Time</td>
<td>T</td>
<td>No Response</td>
</tr>
<tr>
<td>Minimum Detectable Limit</td>
<td>N/A</td>
<td>2.5 M</td>
</tr>
<tr>
<td>[Cholesterol]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Linear Ranges [Cholesterol]</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>Linear Range 1</td>
<td></td>
<td>2.5 – 25 M</td>
</tr>
<tr>
<td>Linear Range 2</td>
<td></td>
<td>45 – 297 M</td>
</tr>
<tr>
<td>Percentage Recovery</td>
<td>N/A</td>
<td>94.3 – 110.5 %</td>
</tr>
</tbody>
</table>
CHAPTER 4

DEVELOPMENT OF A
CHOLESTEROL OXIDASE /
CHOLESTEROL ESTERASE
BIOSENSOR FOR THE
MEASUREMENT OF TOTAL
CHOLESTEROL
CHAPTER 4

DEVELOPMENT OF A CHOLESTEROL OXIDASE/CHOLESTEROL ESTERASE BIOSENSOR FOR MEASUREMENT OF TOTAL CHOLESTEROL

4.1 INTRODUCTION

Most of the cholesterol in human blood and serum (~70%) is esterified with long chain fatty acids, while the remainder occurs as free cholesterol \[3,32,36,40,42,43\]. The determination of total cholesterol (free cholesterol plus cholesterol esters) is essential for the clinical diagnosis of abnormality in lipid metabolism, arteriosclerosis and hypertension \[8-12\]. The link between these disease states and elevated cholesterol levels has been explained in detail in Chapter 1.

The analysis of total cholesterol in human serum is a routine clinical diagnostic test that has been somewhat of a problem for clinical chemists \[244,245\]. A variety of analytical procedures for cholesterol assay have been proposed, including chemical \[246-258\] and enzymatic methods \[259-261\]. In traditional methods, cholesterol is treated to produce coloured reaction products, which are measured spectrophotometrically. The most common reactions are Liebermann-Burchard reaction, the ion salt-sulphonic acid reaction (Kiliani or Zak), and the p-toluene sulfonic acid reaction \[244\]. Extraction and saponification steps precede the colour development \[40\]. Specific disadvantages of these methods are that the colour development
is very dependent on the experimental conditions, suffers from interferences and require very large sample volume \([40,241]\). More significantly, these chemical reactions for total cholesterol determination present certain difficulties, such as lack of specificity and selectivity due to interfering reactions and the use of unstable and/or corrosive reagents.

Since cholesterol esters do not function as substrates for COD, biosensors based on CE have been developed for total cholesterol determination \([185,262-265]\). CE catalyses the hydrolysis of the esters to free cholesterol, which is then detected by various analytical techniques, such as spectrophotometry \([266-273]\), fluorometry \([274]\), amperometry \([275-277]\) and potentiometry \([278]\).

Enzymatic methods for the measurement of total cholesterol therefore appear to be a real improvement when compared to other chemical procedures \([43]\). Since the cholesterol in biological materials, such as blood and serum, is partially esterified with fatty acids, a hydrolytic reaction is required for the assay of total cholesterol \([47,279]\). Hence, COD is used in combination with CE. The basis for all enzymatic cholesterol assays, is the hydrolysis of cholesterol esters (ranging from acetate to stearate) by CE thereby liberating cholesterol (Eqn. 4.1) and the corresponding fatty acids.

\[
\text{cholesterol esters} + \text{H}_2\text{O} \xrightarrow{[\text{CE}]} \text{cholesterol} + \text{fatty acids}
\]  

(Eqn. 4.1)
This is followed by oxidation of free cholesterol to 4-cholesten-3-one by COD with concomitant oxygen consumption, resulting in the production of hydrogen peroxide (H$_2$O$_2$) as illustrated in Eqn. 4.2, below \cite{43,47}.

\begin{equation}
\text{cholesterol} \xrightarrow{\text{COD}} \text{4-cholesten-3-one} + \text{H}_2\text{O}_2
\end{equation}

The immobilisation of COD and CE by cross-linking with BSA and GLA has been previously used \cite{36} for the development of biosensors for determination of cholesterol. However, the cross-linking of the enzymes is not direct cross-linking with BSA-GLA as described in Chapter 3 of this thesis. Yao et. al. \cite{44} have reported on the successful, simultaneous assay of free and total cholesterol in blood serum by FIA. The biosensor is prepared by cross-linking peroxidase with BSA-GLA on a gold sheet. COD and CE are then immobilised onto the columns for the determination of total cholesterol. In Chapter 3 of this thesis, successful results were obtained for the determination of free cholesterol by immobilisation of COD in a BSA-GLA matrix. From our knowledge, this approach has not been previously reported for the determination of total cholesterol. It is therefore of interest for us to investigate whether similar results can be achieved with the co-immobilisation of COD-CE into a BSA-GLA matrix for total cholesterol determination.
In this chapter, the incorporation of cholesterol esterase into a polymeric BSA-GLA matrix will be discussed. The influence of buffer concentration and equilibration time on the sensitivity of the cholesterol response will also be examined. As the ultimate aim of this chapter is to develop a biosensor for total cholesterol determination, co-immobilisation of COD and CE by chemical cross-linking with BSA and GLA will also be investigated. Possible improvement of the performance of the biosensor by use of single layer and bi-layer arrangements will also be explored.

4.2 EXPERIMENTAL

The laboratory conditions, instrumentation, glassware, chemicals and standard solutions, procedures for electrode preparation, pre-treatment and analysis of serum samples, were as, or prepared as described in Section 2.2 of Chapter 2. The cholesterol standard used for the measurement of total cholesterol, contained cholesterol and cholesteryl undec-10-enoate in such amounts that the ratio of free to esterified cholesterol was 30 % (free): 70 % esterified. The procedures for film drying, electrode storage and potentiometric measurement were as, or prepared as described in Section 3.2 of Chapter 3, unless otherwise stated.

4.2.1 Chemicals and Standard Solutions

Cholesterol esterase (CE) EC 3.1.1.13 from pseudomonas flourescens, uric acid and ascorbic acid was purchased from the Sigma-Aldrich Chemical Company. Lyophilised powder containing 100 units of CE was diluted in
200 μL of phosphate buffer solution (pH 7). The enzyme was then divided into 10 μL aliquots that were stored in capped and sealed Eppendorf cells, in the freezer until needed. Stock solutions of 15% w/v BSA and 10% v/v GLA ascorbic acid and uric acid were prepared as described in Section 3.2.1 of Chapter 3.

4.2.2 Procedures

(a) Procedure for Enzyme Immobilisation

(i) Incorporation of CE into BSA-GLA

A 3 μL volume of the mixture as described in Table 4.1 for layer 1 was initially spread onto the platinum electrode and allowed to air dry until the mixture had gelatinised and hardened which took approximately 20 minutes. The electrode was washed under a stream of Milli-Q water to remove any loosely bound molecules, prior to analysis.

Table 4.1:

Composition of CE layer

<table>
<thead>
<tr>
<th>Layer</th>
<th>Vol. 15%BSA (μL)</th>
<th>Vol. 10%GLA (μL)</th>
<th>Vol. CE (μL)</th>
<th>Vol. of Mixture Used (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5 (6.8% w/v)</td>
<td>5 (4.5% v/v)</td>
<td>1* (23 units/mL)</td>
<td>3</td>
</tr>
</tbody>
</table>

* Denotes that amounts were varied during optimisation of parameters
(ii) Co-Immobilisation of COD and CE into BSA-GLA – Single-Layer Film

A 3 μL volume of the mixture as described in Table 4.2 for layer 1 was initially spread onto the platinum electrode and allowed to air dry until the mixture had gelatinised and hardened (~20 minutes). The electrode was washed under a stream of Milli-Q water to remove any loosely bound molecules, prior to analysis.

Table 4.2:

Composition of sensing layer – single layer film

<table>
<thead>
<tr>
<th>Layer</th>
<th>Vol. BSA (μL)</th>
<th>Vol. GLA (μL)</th>
<th>Vol. COD (μL)</th>
<th>Vol. CE (μL)</th>
<th>Vol. of Mixture Used (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5 (6.3% w/v)</td>
<td>5 (4.5% v/v)</td>
<td>1* (21 units/mL)</td>
<td>1* (42 units/mL)</td>
<td>3</td>
</tr>
</tbody>
</table>

* Denotes that amounts were varied during optimisation of parameters.

(iii) Co-Immobilisation of COD and CE into BSA-GLA – Bi-Layer Film

The co-immobilisation of the enzymes in a bi-layer configuration was a two step procedure. A 2 μL volume of the mixture as described in Table 4.3 for layer 1 was initially spread onto the platinum electrode and allowed to air dry until the mixture had gelatinised and hardened (~15-20 minutes). Then 2 μL of the second mixture for layer 2 was spread on top of the first layer. This layer was allowed to air dry until the film had hardened and
gelatinised (~20 minutes). The electrode was washed under a stream of Milli-Q water to remove any loosely bound molecules, prior to analysis.

**Table 4.3:**

**Compositions of sensing layer – bi-layer film**

<table>
<thead>
<tr>
<th>Layer</th>
<th>Vol. BSA (µL)</th>
<th>Vol. GLA (µL)</th>
<th>Vol.COD (µL)</th>
<th>Vol. CE (µL)</th>
<th>Vol. of Mixture Used (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5 (6.8% w/v)</td>
<td>5 (4.5% v/v)</td>
<td>1* (23 units/mL)</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>5 (6.8% w/v)</td>
<td>5 (4.5% v/v)</td>
<td>0</td>
<td>1* (45 units/mL)</td>
<td>2</td>
</tr>
</tbody>
</table>

*Denotes that amounts were varied during optimisation of parameters.

4.3 RESULTS AND DISCUSSION

As mentioned in Chapter 3, the ratio of membrane components is important for the development of a biosensor that is sensitive and reliable. Hence, the concentrations and/or ratios of the CE and COD that are combined to form the sensing layer for both the single and bi-layer configurations need to be optimised. For this reason, the additional factors considered necessary are discussed below. The BSA and GLA components were previously optimised in Chapter 3.
4.3.1 Incorporation of CE into BSA-GLA Film – Optimisation of [CE]

The enzyme concentration in an immobilised matrix affects the response of the biosensor, while the stability of the biosensor is determined by the enzyme loading. The results obtained for varying the [CE] are illustrated in Figure 4.1 and tabulated in Table 4.4. They indicate that the most sensitive response (0.297 ± 0.030 mV/μM) was obtained when 45 units/mL was incorporated into the polymeric matrix. The responses obtained with films formed at this CE concentration were reproducible (MD ± 10.80 %, n = 3), the baselines were smooth and the highest $R^2$ value (0.993) was obtained. This indicates that the response was most linear at this CE concentration. It is possible that at 45 units/mL, an enzyme reserve was built up by incorporating more CE activity in the front of the BSA-GLA matrix, than minimally required to achieve diffusion control \[46\]. In this way, the sensitivity of the biosensor was optimum.

At lower concentrations the response was not as sensitive. This is possibly due to lower enzyme activity, resulting from an insufficient CE reserve in the matrix. At higher concentrations the response appears to decrease and the sensitivity appears to plateau. This is thought to be due to ineffective mass transfer. The highest sensitivities are achieved when there is large enzyme activity within a thin enzyme layer and an effective external mass transfer is provided \[46,235\]. The variation of [CE] in the BSA-GLA-CE biosensor did not influence the achievable linear concentration range for cholesterol, as illustrated in Table 4.4.
Figure 4.1:

Influence of varying [CE] in the BSA-GLA-CE film on the sensitivity of the cholesterol response. Concentrations of components in sensing layer were as described in Table 4.1, except that [CE] was varied. Potentiometric measurement was made in 0.05 M phosphate buffer (pH 7.0) using a 5 mM cholesterol standard solution.
Table 4.4:


<table>
<thead>
<tr>
<th>Film Composition in (μL) Volumes BSA:GLA:CE</th>
<th>Sensitivity (mV/μM)</th>
<th>$R^2$</th>
<th>Linear Range [cholesterol] (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 : 5 : ½</td>
<td>0.116</td>
<td>0.975</td>
<td>24.75 – 99</td>
</tr>
<tr>
<td>5 : 5 : 1</td>
<td>0.2971</td>
<td>0.993</td>
<td>24.75 – 99</td>
</tr>
<tr>
<td>5 : 5 : 2</td>
<td>0.209</td>
<td>0.986</td>
<td>24.75 – 99</td>
</tr>
<tr>
<td>5 : 5 : 4</td>
<td>0.1805</td>
<td>0.987</td>
<td>24.75 – 99</td>
</tr>
<tr>
<td>5 : 5 : 6</td>
<td>0.1753</td>
<td>0.990</td>
<td>24.75 – 99</td>
</tr>
</tbody>
</table>

Corresponding [Concentrations] of Film Composition:

[CE] (units/mL) : ½ μL = 24, 1 μL = 45, 2 μL = 83, 4 μL = 143, 6 μL = 188
[BSA] : 5 μL of BSA = 6.8% w/v
[GLA] : 5 μL of GLA = 4.5% v/v
4.3.2 Effect of Phosphate Buffer Concentration

When cholesterol is oxidised by COD (as shown in equation 4.2) $\text{H}_2\text{O}_2$ is produced. $\text{H}_2\text{O}_2$ is electrochemically active and, hence, can be easily detected. In contrast, the CE hydrolysis reaction illustrated in equation 4.1 does not form an electrochemically active product. Instead, a signal is obtained due to a change in pH caused by the production of fatty acids. For this reason, the optimisation of the buffer concentration needs to be considered in this section. The use of a Pt electrode, which is pH sensitive and the use of potentiometric detection mode is ideal for these conditions.

Figure 4.2 and Table 4.5 show that the maximum sensitivity, 0.233 mV/µM, was obtained in 0.0025 M phosphate buffer (pH 7.0) for the BSA-GLA-CE biosensor for the measurement of cholesterol. The sensitivity appears to decrease slightly past this concentration and tends to plateau. Although 0.0025 M phosphate buffer was the most sensitive, it was also the most time consuming experiment because it took the longest time to establish an equilibrium potential. Figure 4.3 and Table 4.5 show the tendency for the equilibration time to increase at lower phosphate buffer concentrations. A pH-dependant relationship exists within the BSA-GLA-CE film because BSA, GLA and CE are all polyelectrolytes $^{[31]}$. Any change in the pH of the film would also result in a change in the reaction speed. For example, the production of fatty acids in the bulk of the solution would result in a change of pH within the film. At lower buffer concentrations there are possibly an insufficient number of phosphate
Figure 4.2:

Influence of varying [phosphate buffer] on the sensitivity of the cholesterol response obtained with BSA-GLA-CE electrode. Concentrations of components in sensing layer were as described in Table 4.1. Potentiometric measurement was made in varied [phosphate buffer], using a 5 mM cholesterol standard solution.
Figure 4.3:

Influence of varying [phosphate buffer] on the equilibration time of the cholesterol response obtained with BSA-GLA-CE electrode. Conditions were as described for Figure 4.3.
Table 4.5:

Influence of varying [phosphate buffer] on the sensitivity of the cholesterol response obtained with BSA-GLA-CE electrode

<table>
<thead>
<tr>
<th>[Phosphate Buffer] (M)</th>
<th>Sensitivity (mV/µM)</th>
<th>Equilibration Time (minutes)</th>
<th>R²</th>
<th>Linear Range [cholesterol] (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00025*</td>
<td>-</td>
<td>126</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.00125*</td>
<td>-</td>
<td>76</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.0025</td>
<td>0.233</td>
<td>58</td>
<td>0.964</td>
<td>24.75 – 99</td>
</tr>
<tr>
<td>0.005</td>
<td>0.211</td>
<td>42</td>
<td>0.980</td>
<td>24.75 – 99</td>
</tr>
<tr>
<td>0.025</td>
<td>0.203</td>
<td>29</td>
<td>0.994</td>
<td>24.75 – 99</td>
</tr>
<tr>
<td>0.05</td>
<td>0.206</td>
<td>13</td>
<td>0.993</td>
<td>24.75 – 99</td>
</tr>
</tbody>
</table>

* At these buffer concentrations additions over a large concentration range were not completed because the equilibration time in-between each response was 76-126 minutes
ions to neutralise the production of fatty acids, resulting in a drift of the response and a very long stabilisation time in-between additions. Experiments for buffer concentrations 0.25 mM and 1.25 mM had equilibration times of 126 and 76 minutes, respectively, between each response. Overall experiments at these buffer concentrations would be too time consuming and not allow for rapid analysis of samples and for this reason, the experiments were not completed. 0.05 M phosphate buffer (pH 7.0) was chosen as the optimum. Table 4.4 shows that the equilibration time was fastest and the sensitivity (0.206 ± 0.004 mV/μM) was slightly less (~12%) than the maximum sensitivity obtained. At this concentration sufficient phosphate ions are present to neutralise the production of fatty acids, thus resulting in a more reproducible (MD ± 1.70 %, n = 3) response with a smooth baseline and no drift.

4.3.3 Co-Immobilisation of COD and CE into BSA-GLA for Determination of Total Cholesterol in a Single-Layer Film

(a) Optimisation of [COD]

The results obtained for varying the [COD] are illustrated in Figure 4.4 and tabulated in Table 4.6. The achievable linear concentration range for cholesterol was not influenced by variation of [COD]. The optimum response (0.106 ± 0.006 mV/μM) was obtained when 21 units/mL COD was incorporated into the polymeric matrix. The responses obtained with films formed at this COD concentration were fairly reproducible (MD ± 5.40 %, n = 3) and had a low background. The highest R² (0.993) was also
Figure 4.4:

Influence of varying [COD] in BSA-GLA-COD-CE single-layer film on the sensitivity of the response for total cholesterol. Concentrations of components in sensing layer were as described in Table 4.2, except that [COD] was varied. Potentiometric measurement was made in 0.05 M phosphate buffer (pH 7.0) using a 5 mM cholesterol standard solution.
Table 4.6:

Influence of varying [COD] in BSA-GLA-COD-CE single-layer film on the sensitivity of the response for total cholesterol

<table>
<thead>
<tr>
<th>Film Composition in (µL) Volumes</th>
<th>Sensitivity (mV/µM)</th>
<th>R²</th>
<th>Linear Range [cholesterol] (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA:GLA:COD:CE</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 : 5 : ½ : 1</td>
<td>0.079</td>
<td>0.985</td>
<td>49.5 – 297</td>
</tr>
<tr>
<td>5 : 5 : 1 : 1</td>
<td>0.106</td>
<td>0.992</td>
<td>49.5 – 297</td>
</tr>
<tr>
<td>5 : 5 : 1½ : 1</td>
<td>0.076</td>
<td>0.987</td>
<td>49.5 – 297</td>
</tr>
<tr>
<td>5 : 5 : 2 : 1</td>
<td>0.085</td>
<td>0.992</td>
<td>49.5 – 297</td>
</tr>
</tbody>
</table>

**Corresponding [Concentrations] of Film Composition:**

[COD] (units/mL) : ½ µL = 11, 1 µL = 21, 1½ µL = 30, 2 µL = 39
[CE] (units/mL) : 1 µL = 45
[BSA] : 5 µL of BSA = 6.8% w/v
[GLA] : 5 µL of GLA = 4.5% v/v
achieved at this COD concentration indicating that the response was the most linear. At lower concentrations the response was less sensitive perhaps due to lower COD activity in the BSA-GLA-COD-CE film. At higher concentrations the response appears to decrease and the sensitivity appears to plateau. This is thought to be due to ineffective mass transfer \[46\]. Another possibility for the decrease in the sensitivity at higher COD concentrations is proximity problems. The enzymes are probably being cross-linked to each other causing denaturing and loss of enzyme activity \[46,184,207\].

(b) Optimisation of [CE]

The incorporation of 42 units/mL of CE into the polymeric matrix of BSA-GLA-COD gave the optimum cholesterol response. This is illustrated in Figure 4.5 and tabulated in Table 4.7. The highest linearity $R^2$ (0.993) was obtained with films formed at this CE concentration. At lower concentrations the response was not as sensitive. This is possibly due to an insufficient amount of CE in the BSA-GLA-COD-CE film thus decreasing the sensitivity of the sensor. At higher concentrations, the response appears to decrease and the sensitivity appears to plateau in a similar pattern as the responses obtained at the higher concentrations of COD. Another possibility for the decrease in sensitivity, at higher CE concentrations is proximity problems which is associated with excess enzyme cross-linking, for the same reasons described above at higher COD concentrations \[46,184,207\]. The linear concentration range for the BSA-GLA-COD-CE biosensor for total cholesterol measurement was not influenced by variation of [CE].
Figure 4.5:

Influence of varying [CE] in BSA-GLA-COD-CE single-layer film on the sensitivity of response for total cholesterol. Concentrations of components in sensing layer were as described in Table 4.2 except [CE] was varied. Potentiometric measurement was made in 0.05 M phosphate buffer (pH 7.0) using a 5 mM cholesterol standard solution.
Table 4.7:


<table>
<thead>
<tr>
<th>Film Composition in (µL) Volumes</th>
<th>Sensitivity (mV/µM)</th>
<th>R²</th>
<th>Linear Range [cholesterol] (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA:GLA:COD:CE</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 : 5 : 1 : ½</td>
<td>0.071</td>
<td>0.973</td>
<td>49.5 – 297</td>
</tr>
<tr>
<td>5 : 5 : 1 : 1</td>
<td>0.106</td>
<td>0.992</td>
<td>49.5 – 297</td>
</tr>
<tr>
<td>5 : 5 : 1 : 1½</td>
<td>0.091</td>
<td>0.995</td>
<td>49.5 – 297</td>
</tr>
<tr>
<td>5 : 5 : 1 : 2</td>
<td>0.087</td>
<td>0.994</td>
<td>49.5 – 297</td>
</tr>
</tbody>
</table>

Corresponding [Concentrations] of Film Composition:

[CE] (units/mL) : ½ µL = 22, 1 µL = 42, 1½ µL = 60, 2 µL = 77

[COD] (units/mL) : 1 µL = 23

[BSA] : 5 µL of BSA = 6.8% w/v

[GLA] : 5 µL of GLA = 4.5% v/v
4.3.4 Co-Immobilisation of COD and CE into BSA-GLA in a Bi-Layer Film

Table 4.8 gives the composition of the membrane components used to construct the chemically cross-linked bi-layer bi-enzyme biosensor. The optimum concentration for COD in layer 1 was found to be 23 units/mL. Table 3.3 and Figure 3.6 show that at this COD concentration the highest sensitivity was obtained. The optimum concentration for CE in layer 2 was found to be 45 units/mL. Table 4.4 and Figure 4.1 shows that at this CE concentration the highest sensitivity was obtained. The bi-layer was configured so that the CE enzyme layer would be on the outside, and the COD enzyme layer would be on the inside. It is necessary for the CE to be in contact with the substrate first so that it is able to catalyse the hydrolysis of the cholesterol fatty acid esters (equation 4.1) to free cholesterol, which can then be oxidised by the COD (equation 4.2).

**Table 4.8:**

Optimised compositions of sensing layers for bi-layer configuration

<table>
<thead>
<tr>
<th>Layer</th>
<th>[BSA] (w/v)</th>
<th>[GLA] (v/v)</th>
<th>[COD] (units/mL)</th>
<th>[CE] (units/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.8% w/v</td>
<td>4.5% v/v</td>
<td>23</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>6.8% w/v</td>
<td>4.5% v/v</td>
<td>0</td>
<td>45</td>
</tr>
</tbody>
</table>
4.3.5 Comparison of Single-Layer and Bi-Layer Film Configurations

Figure 4.6 and Table 4.9 show the influence of single or bi-layer film configuration, on the sensitivity of bi-enzyme BSA-GLA-COD/BSA-GLA-CE biosensor for total cholesterol determination. The bi-layer film appears to have a better performance. It gave a more sensitive response as well as a slightly higher $R^2$ value, which indicates that the response is more linear. An added advantage of the bi-layer arrangement is the ability to control the location of enzyme. As mentioned previously, with cholesterol the CE needs to be on the outside to catalyse the first hydrolysis step. In the configuration of a single layer film, it is not possible to control the location of each of the enzymes because they are simply mixed into the polymeric matrix created by the BSA and GLA. Hence, the available amount of the enzyme for each reaction step is not optimum with the use of a single layer arrangement.

4.3.6 Interference Study

Ascorbic and uric acid are the two most common interferents that affect the sensitivity of cholesterol biosensors [40]. Therefore, it is important that the cholesterol biosensor should have high specificity and be able to discriminate against these coexisting substances [54,241,242]. Alternatively, methods need to be developed to suppress the interference caused by these contaminants. It is necessary to investigate the effect of these interferents on the response, before developing methods to suppress them.
Figure 4.6:

Influence of single and bi-layer film configurations on the sensitivity of the cholesterol responses of BSA-GLA-COD-CE and BSA-GLA-COD/BSA-GLA-CE biosensors. Concentration of components in sensing layer for the single layer and bi-layer response were as described in Table 4.1 and 4.2 respectively. Potentiometric measurement was made in 0.05 M phosphate buffer (pH 7.0) using a 5 mM cholesterol standard solution.
Table 4.9:

Influence of single or bi-layer film configuration on the sensitivity of the combined enzyme biosensors for total cholesterol measurement

<table>
<thead>
<tr>
<th></th>
<th>Single-Layer Film BSA-GLA-COD-CE</th>
<th>Bi-Layer Film BSA-GLA-COD/BSA-GLA-CE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sensitivity</strong></td>
<td>0.106</td>
<td>0.1451</td>
</tr>
<tr>
<td>(mV/µM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>R²</strong></td>
<td>0.992</td>
<td>0.997</td>
</tr>
<tr>
<td><strong>Linear Range</strong></td>
<td>49.5 – 297</td>
<td>49.5 – 297</td>
</tr>
<tr>
<td>[cholesterol] (µM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Enzyme Arrangement</strong></td>
<td>COD and CE combined in single layer</td>
<td>CE: Layer 2 (outside) COD: Layer 1 (inside)</td>
</tr>
</tbody>
</table>

**Corresponding [Concentrations] of Single and Bi-Layer Film Composition:**

[CE] (units/mL) = 45
[COD] (units/mL) = 23
[BSA] : 5 µL of BSA = 6.8% w/v
[GLA] : 5 µL of GLA = 4.5% v/v
(a) **Effect of Ascorbic Acid**

As discussed in Chapter 3, ascorbic acid is a major interferent to cholesterol biosensors. Figure 4.7 and Table 4.10 show that the presence of ascorbic acid interferes with the response significantly. The addition of 0.001 mM ascorbic acid results in a significant 27.8% decrease in the sensitivity of the response. Table 4.10 shows that this suppression effect increases in the presence of higher ascorbic acid concentrations, to the extent where 0.1 mM ascorbic acid causes an 84.6% suppression in the response. 1 mM ascorbic acid results in a 100% suppression. The background was very noisy and the potential did not stabilise at these high concentrations. As discussed in Chapter 3 the presence of high concentrations of ascorbic acid in the solution results in the oxidation of the ascorbic acid with the hydrogen peroxide, hence decreasing the sensitivity of the response [54]. The reported concentrations of ascorbic acid in serum ranges from 45 – 90 μM [3] and at these concentrations the ascorbic acid could suppress the response between 61.9 – 84.6% as, indicated in Table 4.10. Usually, this effect of ascorbic acid on the response would be of concern if analyses were being conducted on undiluted serum. However, as mentioned in Chapter 3 during this course of study, serum was analysed using a 1:200 dilution, for reasons mentioned in Chapter 3. Hence, it is again unlikely that at these high dilutions of serum, the presence of ascorbic acid between the range of 0.23 – 0.45 μM will affect the response obtained for cholesterol in this sample, with the BSA-GLA-COD/BSA-GLA-CE biosensor.
Figure 4.7:

Influence of ascorbic acid on the sensitivity of the cholesterol response obtained with the bi-layer BSA-GLA-COD/BSA-GLA-CE biosensor. Concentration of components in sensing layer were as described in Table 4.3. Potentiometric measurement was made in 0.05 M phosphate buffer (pH 7.0) using a 5 mM cholesterol standard solution.
Table 4.10:

Influence of [ascorbic acid] on the sensitivity of the cholesterol response obtained with the bi-layer BSA-GLA-COD/BSA-GLA-CE biosensor

<table>
<thead>
<tr>
<th>[Ascorbic Acid] (mM)</th>
<th>Sensitivity (mV/ M)</th>
<th>Percentage of Response Suppressed (%)</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.106</td>
<td>0</td>
<td>0.993</td>
</tr>
<tr>
<td>0.001</td>
<td>0.076</td>
<td>27.8</td>
<td>0.984</td>
</tr>
<tr>
<td>0.01</td>
<td>0.04</td>
<td>61.9</td>
<td>0.931</td>
</tr>
<tr>
<td>0.1</td>
<td>0.016</td>
<td>84.6</td>
<td>0.945</td>
</tr>
<tr>
<td>1</td>
<td>No Response</td>
<td>100</td>
<td>N/A</td>
</tr>
</tbody>
</table>
(b) **Effect of Uric Acid**

As mentioned previously uric acid is also classified as a common interferent but is sometimes not as limiting as ascorbic acid \(^4\). Figure 4.8 and the results in Table 4.11, illustrate that the presence of uric acid reduces the signal of the biosensor and, hence, there is a decrease in sensitivity. At the lower uric acid concentrations of 0.001 – 0.01 mM, the response was affected by a decrease in sensitivity between ~31 – 33.9%. This decrease in sensitivity is much more apparent at higher uric acid concentrations where between 0.1 – 1 mM there is an 83.3 – 100% suppression in the response.

The presence of a very high concentration of uric acid in the measurement solution favours the oxidation of uric acid with the hydrogen peroxide, and thereby suppressing the response of the cholesterol measurement \(^5\). The responses were very similar to those obtained at very high ascorbic acid concentrations.

The presence of uric acid does interfere with the response of the BSA-GLA-COD/BSA-GLA-CE biosensor to cholesterol, and should be cause for concern, because the reported levels of uric acid in serum range between 140 – 420 \(\mu\)M \(^3\). However, with the 1:200 dilution being used in the course of this work, the level of uric acid present in diluted serum is 0.7 – 2.1 \(\mu\)M. It is unlikely that there would be any suppression in the response by uric acid at these low concentrations.
Figure 4.8:

Influence of [uric acid] on the sensitivity of the cholesterol response obtained with the bi-layer BSA-GLA-COD/BSA-GLA-CE biosensor. Conditions were as described in Figure 4.7.
Table 4.11:

Influence of [uric acid] on the sensitivity of the cholesterol response obtained with the bi-layer BSA-GLA-COD/BSA-GLA-CE biosensor

<table>
<thead>
<tr>
<th>[Uric Acid] (mM)</th>
<th>Sensitivity (mV/ M)</th>
<th>Percentage of Response Suppressed (%)</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.171</td>
<td>0</td>
<td>0.990</td>
</tr>
<tr>
<td>0.001</td>
<td>0.113</td>
<td>33.9</td>
<td>0.989</td>
</tr>
<tr>
<td>0.01</td>
<td>0.118</td>
<td>31</td>
<td>0.992</td>
</tr>
<tr>
<td>0.1</td>
<td>0.029</td>
<td>83.3</td>
<td>0.986</td>
</tr>
<tr>
<td>1</td>
<td>No Response</td>
<td>100</td>
<td>N/A</td>
</tr>
</tbody>
</table>
4.3.7 Linear Concentration Range and Minimum Detectable Amount

The minimum detectable amount of cholesterol with the combined enzyme bi-layer BSA-GLA-COD/BSA-GLA-CE biosensor, fabricated by chemical cross-linking, was 2.5 \( \mu \text{M} \). Figure 4.9 (a) illustrates that the first achievable linear concentration range for cholesterol with this biosensor was between 2.5 – 32 \( \mu \text{M} \) cholesterol and the second linear range appeared to be between 49.5 – 297 \( \mu \text{M} \) as shown in Figure 4.9 (b). The first linear range with a slope of 0.4323 mV/\( \mu \text{M} \) appeared to be more sensitive than the second linear range with a slope of 0.1086 mV/\( \mu \text{M} \).

The presence of two linear ranges at such different cholesterol concentrations implies that sensitive quantification of cholesterol can be achieved at the low levels, while more concentrated cholesterol solutions can be determined at the higher levels.

As mentioned previously the reproducibility of fabrication of an electrode is important. For this reason the sensitivity of the BSA-GLA-COD/BSA-GLA-CE biosensor over several different fabrications was analysed. The achieved sensitivity (0.1129 \( \pm \) 19.6 \%) mV/\( \mu \text{M} \) indicates that the BSA-GLA-COD/BSA-GLA-CE biosensor sensitivities given, were fairly consistent with repeated use of the biosensor over several different fabrications.
Figure 4.9 (a):

Calibration curve obtained for the bi-layer BSA-GLA-COD/BSA-GLA-CE biosensor. The “Linear Range 1” curve shows that the sensor displays a linear response in the range of \([2.5 \text{ – } 32] \mu\text{M}\) cholesterol.
Figure 4.9 (b):

Calibration curve obtained for the bi-layer BSA-GLA-COD/BSA-GLA-CE biosensor. The "Linear Range 2" curve shows that the sensor displays a linear response in the range of [49.5 – 297] μM cholesterol.
4.3.8 Percentage Recovery and Application to Serum Samples

Table 4.12 shows that excellent recoveries, in the range of 98.89 - 103.71 μM cholesterol were achieved with combined enzyme bi-layer BSA-GLA-COD/BSA-GLA-CE biosensor for the quantification of known spiked amounts of cholesterol. These results demonstrate that accurate and sensitive determination of cholesterol can be accomplished with the biosensor. The standard deviation values also demonstrate that the sensor allows for reproducible quantification.

A typical potentiogram obtained for quantification of cholesterol in serum with the bi-layer BSA-GLA-COD/BSA-GLA-CE biosensor is illustrated in Figure 4.10. It shows that the response to the addition of serum took longer to reach equilibrium, whereas the response to the additions of the standard stabilised more quickly. This may be due to some matrix effect of the serum. The performance of the biosensor in the analysis of blood serum cholesterol is also shown in Table 4.13. The values obtained for the determination of cholesterol were similar to the values obtained by the standard colorimetric method used at the Royal Prince Alfred Hospital. The results obtained with the BSA-GLA-COD/BSA-GLA-CE biosensor are more similar to the results obtained by the clinical method than the results obtained with the BSA-GLA-COD. Therefore, this biosensor is more accurate and sensitive for the determination of cholesterol in serum samples, than the BSA-GLA-COD biosensor in Chapter 3.
Table 4.12:

Sensitivity of bi-layer BSA-GLA-COD/BSA-GLA-CE biosensor in percentage recovery of total cholesterol.

<table>
<thead>
<tr>
<th>[Total Cholesterol Added] (µM)</th>
<th>[Cholesterol Recovered] (µM)</th>
<th>Percentage Recovery (MD, n = 3) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.19</td>
<td>6.12</td>
<td>98.89 ± 1.13</td>
</tr>
<tr>
<td>12.38</td>
<td>12.84</td>
<td>103.71 ± 3.6</td>
</tr>
<tr>
<td>24.75</td>
<td>24.55</td>
<td>99.19 ± 0.8</td>
</tr>
<tr>
<td>49.50</td>
<td>49.47</td>
<td>99.94±1.35</td>
</tr>
</tbody>
</table>
Figure 4.10:

Typical potentiometric responses obtained for the quantification of cholesterol in serum with the BSA-GLA-COD/BSA-GLA-CE biosensor. Cholesterol concentrations: (Std. 1) 6.2 ; (Std. 2) 12.4 and (Std. 3) 18.6 μM. Concentration of components in sensing layer were as described in Table 4.3.
Table 4.13:

Determination of cholesterol in serum samples with a bilayer BSA-GLA-COD/BSA-GLA/CE biosensor

<table>
<thead>
<tr>
<th>Sample Number</th>
<th>[Cholesterol] measured by Clinical Method (mM)</th>
<th>[Cholesterol] measured by Biosensor (MD, n = 3) (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ser/P1 5006-4</td>
<td>4.09</td>
<td>3.96 ± 0.30</td>
</tr>
<tr>
<td>Ser/P1 5009-1</td>
<td>4.15</td>
<td>4.55 ± 1.45</td>
</tr>
<tr>
<td>Ser/P1 5006-2</td>
<td>4.04</td>
<td>3.68 ± 0.14</td>
</tr>
</tbody>
</table>
4.4 CONCLUSION

Initially CE was successfully incorporated into a GLA-BSA polymeric matrix. Both single layer and bi-layer arrangements of the BSA-GLA-COD/BSA-GLA-CE biosensor enabled accurate potentiometric measurement of total cholesterol. More sensitive responses were obtained with the bi-layer configuration. Hence, the BSA-GLA-COD/BSA-GLA-CE biosensor was chosen for further experimental work. An added advantage of the bi-layer arrangement is the ability to immobilise the CE in the outside layer to catalyse the first hydrolysis step. It is not possible to control the location of each of the enzymes in the single-layer film configuration because the COD and the CE are simply mixed into the BSA-GLA polymeric matrix.

The achievable minimum detectable amount of cholesterol with this BSA-GLA-COD/BSA-GLA-CE biosensor is 2.5 μM and a linear concentration range of 2.5 – 32 μM was achieved in the most sensitive range. Recovery studies gave excellent results with percentage recoveries in the range (98.89 - 103.71%). The biosensor was successfully applied to serum samples.
4.5 SUMMARY OF RESULTS

Table 4.14:
Results and optimum conditions obtained with a BSA-GLA-COD/BSA-GLA-CE biosensor for total cholesterol measurement

<table>
<thead>
<tr>
<th>PARAMETER</th>
<th>OPTIMUM CONDITION / RESULT</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Bovine Serum Albumin]</td>
<td>6.8 % w/v</td>
</tr>
<tr>
<td>[Glutaraldehyde]</td>
<td>4.5% v/v</td>
</tr>
<tr>
<td>[Cholesterol Oxidase] Inner-Layer 1</td>
<td>23 units/mL</td>
</tr>
<tr>
<td>[Cholesterol Esterase] Outer-Layer 2</td>
<td>45 units/mL</td>
</tr>
<tr>
<td>[Phosphate Buffer]</td>
<td>0.05 M</td>
</tr>
<tr>
<td>Minimum Detectable Amount</td>
<td></td>
</tr>
<tr>
<td>[Cholesterol]</td>
<td>2.5 M</td>
</tr>
<tr>
<td>Linear Ranges [Cholesterol]</td>
<td></td>
</tr>
<tr>
<td>Linear Range 1</td>
<td>2.5 – 32 M</td>
</tr>
<tr>
<td>Linear Range 2</td>
<td>49.5 – 297 M</td>
</tr>
<tr>
<td>Percentage Recovery</td>
<td>98.9 – 103.7 %</td>
</tr>
</tbody>
</table>
CHAPTER 5

DEVELOPMENT OF A HYBRID POLYPYRROLE BASED CROSS-LINKED CHOLESTEROL OXIDASE BI-LAYER BIOSENSOR FOR THE MEASUREMENT OF FREE CHOLESTEROL
CHAPTER 5

DEVELOPMENT OF A HYBRID POLYPYRROLE-BASED/CROSS-LINKED COD BI-LAYER BIOSENSOR FOR CHOLESTEROL

5.1 INTRODUCTION

Polymer multilayers have been used to expand the analytical scope of single layer biosensors. They provide a number of alternatives for the improvement of sensitivity by means of the design of layers that increase the efficiency of electron transfer between the enzyme and the electrode \cite{280,281}. Multilayer configurations are of particular interest since they provide several possible alternatives for improving selectivity. They exhibit a much higher degree of interference rejection by the combination of the permselective qualities of several polymers, than when each polymer is separate \cite{32,280}. Furthermore, multilayers solve the problem of enzyme entrapment when the enzymes cannot be entrapped in the polymer layer, because the polymerisation conditions have an adverse effect on the activity of the enzyme \cite{280,281}. They are also useful when the biosensor does not produce satisfactory results because of enzyme polymer steric factors or density problems associated with the substrate \cite{282}.

Significantly improved sensitivity was obtained with multi-layer configurations \cite{284-285} due to an increase in the effective area of the electrode, which permitted higher enzyme loading, and enhanced detection of the enzymatically generated product \cite{34}.
In order to obtain greater selectivity, alternative configurations were designed which either make it possible to enhance the exclusion of the polymer layer or improve analytical properties, such as biosensor sensitivity and stability [33].

PPy has a porous structure, therefore it has useful properties for the exclusion of interferences [286]. These size exclusion properties can be improved if the polymer is overoxidised at a potential higher than 0.7 V. The irreversible overoxidation of PPy film produces a non-conducting film with remarkable permselectivity stemming from size and anion-exclusion properties. This is due to the permeability of the polymer and to the generation of anionic groups in the polymer backbone with high electron density that act as a barrier against the diffusion of anionic species into the film [283,287].

The electrochemical immobilisation of enzymes has some drawbacks, which limits its use for fabrication of biosensors. Some enzymes may be incompatible with certain polymeric matrices or with the chemical environment generated at the electrode surface during the electropolymerisation [283,287]. Furthermore, electrochemical immobilisation of enzymes seems only successful with enzymes exhibiting significant non-denaturing adsorption onto or entrapment into the electrode surface [289,290]. Even when thicker layers are obtained by immobilisation with conducting polymers, the incorporation of the enzyme into the growing film requires electrostatic affinity between the polymer and the enzyme so that, for example, positively charged proteins cannot be entrapped in PPy films [283].
The long-term stability of these biosensors is significantly reduced by the ageing of the enzyme-entrapped polymer (ie: the modification of its diffusion/partition characteristics). A low amount of immobilised enzyme (typically in a monolayer) also tends to reduce the long-term stability of the biosensor [291]. A way of overcoming these problems could be utilising a hybrid biosensor design. This method allows the advantages of electrosynthesised non-and/or conducting polymers to be coupled with those of a classical immobilisation method, such as chemical cross-linking using BSA and GLA [291]. In this way, the permselective qualities of a multilayer configuration are coupled with the high enzyme loading and long term stability of cross-linking with BSA and GLA [291,292]. The use of this electrode configuration has not been reported for the determination of cholesterol, but Guerrieri et. al. [291] have used the method for the development of a GOD-overoxidised PPy bilayer electrode. GOD was cross-linked in a BSA-GLA matrix, on top of a pre-polymerised overoxidised PPy layer. This method was successful for the fabrication of an electrode for the sensitive determination of glucose. The advantages of this method, listed previously, were the driving force for the research described in this chapter to develop a similar biosensor for the detection of cholesterol using a similar method as Guerrieri et. al. [291] except in this case immobilising COD in the BSA-GLA.

The aim of the research outlined in this section is to design a hybrid bilayer configuration with layer 1 consisting of an electropolymerised PPy layer and layer 2 consisting of COD cross-linked by BSA and GLA for free cholesterol determination. The specific objectives of this chapter are to:
(a) Evaluate the influence of the different types of films for layer 1, such as overoxidised PPy, conducting polypyrrole-nitrate and polypyrrole-chloride for biosensing of cholesterol;
(b) Assess the effect of polymerisation time on the sensitivity of the cholesterol response, as well as the dependence of the sensitivity of the biosensor on the thickness of the BSA-GLA-COD layer 2;
(c) Use the optimum conditions to construct a bilayer PPy-NO₃/BSA-GLA-COD biosensor for accurate and sensitive measurement of cholesterol.

5.2 EXPERIMENTAL

Laboratory conditions, glassware, instrumentation, cholesterol standards and procedures for electrode preparation, pre-treatment and analysis of serum samples, were as or prepared as described in Section 2.2 of Chapter 2. Pyrrole was distilled as described in Section 2.2.4 in Chapter 2. Chemicals and standard solutions, procedures for film drying of layer 2, electrode storage and potentiometric measurement, were as or prepared as described in Section 3.2 of Chapter 3, unless otherwise stated.

5.2.1 Procedure for Electrode Preparation

(a) Preparation of Bi-Layer Film

The preparation of the biosensor was a two-step procedure. Each step represents the formation of each layer.
(i) Layer 1 – Polypyrrole – Chloride (PPy-Cl) Film

The galvanostatic electropolymerisation of the PPy-Cl film was performed using a three-electrode voltammetric cell. The working electrode was a platinum electrode (0.03 cm²), whilst a platinum wire and an Ag/AgCl were used as the auxiliary and reference electrodes, respectively. The PPy-Cl film was formed in a solution containing 0.1 M pyrrole and 0.1 M KCl, with an applied current density of 0.25 mA/cm² for either 60 or 300 s. After the galvanostatic film formation, the polymer electrode was washed several times under a stream of Milli-Q water to remove remaining monomer solution. It was shaken gently and the sides were dried with fibre-free tissue, to remove excess water. The electrode was then fixed to a retort stand in preparation for the next step.

(ii) Layer 1 – Polypyrrole – Nitrate (PPy-NO₃) Film

The galvanostatic electropolymerisation of the PPy-NO₃ film was prepared as described in 5.2.1 (a) (i) of this chapter, except the PPy-NO₃ film was formed in a monomer solution containing 0.1 M pyrrole and 0.1 M KNO₃.

(iii) Layer 1 – Overoxidised PPy-NO₃ Film

The PPy-NO₃ film was prepared as described above in 5.2.1 (a) (ii) of this chapter. Instead of fixing the electrode to a stand, it was placed in a cell containing 0.05 M phosphate buffer (pH 7.0). The film was then overoxidised by application of a potential of 1 V for of 20 minutes or until the current decreased below 10⁻⁵ mA.
(iv) Layer 2 – Incorporation of COD into BSA-GLA

3 μL of the mixture for layer 2 (optimised in Chapter 3) as described in Table 5.1, was spread onto the polymerised PPy layer 1. Layer 2 was allowed to air dry until the film mixture had gelatinised and hardened. The electrode was washed under a stream of Milli-Q water to remove any loosely bound molecules, prior to analysis.

Table 5.1:
Composition of layer 2 in PPy-NO$_3$/BSA-GLA-COD biosensor

<table>
<thead>
<tr>
<th>Layer</th>
<th>Vol. BSA (μL)</th>
<th>Vol. GLA (μL)</th>
<th>Vol. CE (μL)</th>
<th>Vol. of Mixture Used (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>5 (6.8% w/v)</td>
<td>5 (4.5% v/v)</td>
<td>1 (23 units/mL)</td>
<td>3</td>
</tr>
</tbody>
</table>

5.3 RESULTS AND DISCUSSION

5.3.1 Characterisation of PPy Films by Chronopotentiometry and Cyclic Voltammetry

(a) Chronopotentiometry

As discussed in Chapter 2 the technique of chronopotentiometry can be used to investigate the conductivity and ease of polymerisation of PPy films. Figure 5.1 illustrates the typical chronopotentiograms obtained for
the polymerisation of (a) PPy-NO₃ and (b) PPy-Cl films, while Figure 5.2 illustrates typical chronopotentiograms obtained for (a) PPy-NO₃, (b) overoxidised PPy-NO₃ films. Generally, conductive polymer materials have a low initial potential for polymerisation, and the potential either remains low or decreases during the polymerisation.

Figure 5.1 (a) shows that the PPy-NO₃ film had a low initial potential of 319 mV, increased to a maximum of ~626 mV then decreased before finally stabilising at a potential of ~587 mV. The low initial potential indicates that the polymer film was easy to polymerise and the low stabilising potential indicates that the polymer was very conductive.

Figure 5.1 (b) shows that the chronopotentiogram for the PPy-Cl film was similar to that obtained for the PPy-NO₃ film described above. The PPy-Cl film had an initial polymerisation potential of 477 mV. The potential increased during the film formation, reached a maximum at ~646 mV and finally stabilising at a potential of ~627 mV. The PPy-Cl film fabricated by this method is also conductive and easy to polymerise, for the reasons discussed above.

Figure 5.2 (a) is a chronopotentiogram of a PPy-NO₃ film prior to overoxidation. The characteristic of the film is very similar to the PPy-NO₃ film in Figure 5.1 (a). The initial potential for this film was 325 mV, the maximum potential was ~618 mV and the stabilising potential of ~564 mV. This film was easy to polymerise and conductive because the initial, maximum and stabilising potential remained low during the electropolymerisation. The potential values obtained for the PPy-NO₃ film were slightly lower than those obtained for the PPy-Cl film.
Figure 5.1:

Characterisation of (a) PPy-NO$_3$ and (b) PPy-Cl films by chronopotentiometry: Conditions for (a): 0.3 M pyrrole, 0.1 M KNO$_3$, 0.5 mA/cm$^2$ for 60 s.

(b): as described above except 0.1 M KCl was added instead of KNO$_3$. 
Figure 5.2:

Characterisation of (a) PPy-NO$_3$ and (b) overoxidised PPy-NO$_3$ films by chronopotentiometry:

Conditions for (a): 0.3 M pyrrole, 0.1 M KNO$_3$, 0.5 mA/cm$^2$ for 60 s.
(b) as described above except film was overoxidised by application of 1 V potential for 20 minutes (or until the current decreased below 1x10$^{-5}$ mA).
indicating that in this case PPy-NO$_3$ film was slightly more conductive than the PPy-Cl film.

Figure 5.2b shows that the conductivity of the PPy-NO$_3$ film decreased considerably when the film was overoxidised by the application of a potential of 1 V for twenty minutes. At this potential the current decreased continuously over the period of twenty minutes. After this period the potential decreased below $1\times10^{-5}$ mA indicating that the PPy-NO$_3$ film was completely overoxidised and non-conductive.

(b) Cyclic Voltammetry

The CV obtained for a PPy-NO$_3$ film in 0.1 M sodium nitrate (NaNO$_3$) is very similar to the CV illustrated in Figure 2.2 (a). The characteristic oxidation and reduction couple of PPy appeared at approximately $-0.1$ and $-0.15$ V versus Ag/AgCl, respectively. Figure 5.3 (a) is the CV obtained for the PPy-Cl film in 0.01 M NaNO$_3$. The characteristic oxidation and reduction couple of PPy appeared at approximately $-0.3$ and $-0.35$ V versus Ag/AgCl respectively.

Figure 5.3 (b) is the CV obtained for overoxidised PPy-NO$_3$ in 0.1 M sodium nitrate. The characteristic oxidation and reduction couple of PPy is absent. There is a very small reduction peak between $-0.4$ and $-0.5$ V versus Ag/AgCl, however the peak has shifted by $\sim100$ mV in the negative direction possibly due to a decrease in the conductivity of the film. There is no oxidation peak present. The CV for the overoxidised PPy-NO$_3$ indicates that the film is non-conductive and that the reaction is irreversible.
Figure 5.3:
Characterisation of (a) PPy-Cl and (b) overoxidised PPy-NO₃ films by cyclic voltammetry in 0.1 M NaNO₃ at scan rate of 50 mV/s. Scanning Range: Initial Potential +400 mV to -800 mV. Polymerisation conditions for 5.3(a) and 5.3 (b) were as described in Figure 5.1 (b) and Figure 5.2 respectively.
5.3.2 Dependence of Sensitivity on Type of Pyrrole Film - Layer 1

The results in Figure 5.4 and Table 5.2 illustrate that irrespective of the type of pyrrole film the response of the biosensor to cholesterol was much higher at the lower polymerisation time of 60 s than at 300 s. In fact, the sensitivity of the response decreased significantly with increasing polymerisation time beyond a period of 60 s. This may be due to the increased thickness of the PPy film, which limits the rate at which the catalytic product can be detected at the platinum electrode due to the increased diffusion barrier. In addition, at longer polymerisation times the cholesterol response was not as reproducible and the equilibration time inbetween additions was longer. The longer response times are indicative of an increase in the diffusion barrier, thus changing the rate of the reaction and increasing the response time. A polymerisation time of 60 s was therefore used for all subsequent work.

The use of overoxidised PPy films has been shown to exhibit excellent permselective and interference rejection qualities in previous reported work on cholesterol biosensors [32-34,226]. Figure 5.4 shows that biosensors based on overoxidised PPy-NO₃ films were less sensitive than their conducting electropolymerised counterparts. It is possible that the lack of electrical conductivity makes the film less responsive to the catalytic product generated in the outer layer [282]. Figure 5.4 shows that for layer 1 the PPy-NO₃ polymerised for 60 s were the most sensitive (0.150 ± 0.005 x mV/µM) Table 5.2 also shows that it had the highest R² value and best linear range combination.
Figure 5.4:

Influence of type of pyrrole film in layer 1 on the sensitivity of the cholesterol response obtained with bi-layer PPy-NO\(_3\)/BSA-GLA-COD. Concentrations of components in sensing layer were as described in Table 5.1. Potentiometric measurement was made in 0.05 M phosphate buffer (pH 7.0) and a 5 mM cholesterol standard solution was used.

**Key:**
- **Overoxidised PPy Film:**
- **Conducting PPy-Cl Film:**
- **Conducting PPy-NO\(_3\) Film:**
Table 5.2:
Influence of type of PPy-film in layer 1 on the performance of PPy-NO₃/BSA-GLA-COD bi-layer biosensor for free cholesterol measurement

<table>
<thead>
<tr>
<th>Type of PPy Film (Layer 1)</th>
<th>Polymerisation Time (s)</th>
<th>Sensitivity (mV/ M)</th>
<th>R²</th>
<th>Linear Range [cholesterol] (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPy-Cl</td>
<td>60</td>
<td>0.13</td>
<td>0.983</td>
<td>49.5 – 247.5</td>
</tr>
<tr>
<td>PPy-Cl</td>
<td>300</td>
<td>0.08</td>
<td>0.999</td>
<td>99 – 247.5</td>
</tr>
<tr>
<td>PPy-NO₃</td>
<td>60</td>
<td>0.15</td>
<td>0.994</td>
<td>49.5 – 247.5</td>
</tr>
<tr>
<td>PPy-NO₃</td>
<td>300</td>
<td>0.07</td>
<td>0.971</td>
<td>49.5 – 247.5</td>
</tr>
<tr>
<td>* PPy-NO₃ (ox)</td>
<td>60</td>
<td>0.11</td>
<td>0.984</td>
<td>148.5 – 247.5</td>
</tr>
<tr>
<td>* PPy-NO₃ (ox)</td>
<td>300</td>
<td>0.06</td>
<td>0.995</td>
<td>49.5 – 247.5</td>
</tr>
</tbody>
</table>

* (ox) denotes overoxidised film
A thinner film is desirable due to expected faster response times and higher sensitivity\(^{[282]}\). For these reasons, PPy-NO\(_3\) polymerised for 60 s was chosen as the optimum for layer 1.

### 5.3.3 Dependence of Sensitivity on Layer 2 Mixture Volume

The thickness of the BSA-GLA-COD in layer 2 affects the response of the bilayer PPy-NO\(_3\)/BSA-GLA-COD biosensor. The results obtained for varying the volume of the mixture used for layer 2 and hence, the thickness of layer 2 (as described in Table 5.1), are illustrated in Figure 5.5 and Table 5.3. These results show that the most sensitive response (0.099 ± 5 x 10\(^{-4}\) mV/µM) was obtained when 2 µL of the mixture was spread on top of the PPy-NO\(_3\) (layer 1). The responses obtained with films formed at this film thickness were reproducible (MD ±

\[ \% \text{, } n = 3 \]), the baselines were fairly smooth and the highest R\(^2\) value (0.994) was obtained. This indicates that the response obtained at this film thickness was the most linear, with a linear range between 49.5 – 247.5 µM cholesterol. Films produced with less than 2 µL of the mixture had a less sensitive response. This is possibly due to lower enzyme activity resulting from the presence of an insufficient enzyme reserve in the matrix. To a major extent the enzyme loading in immobilised matrices determines the stability of the biosensor, as mentioned previously. An enzyme reserve is built up by employing more enzyme activity in the front of the electrochemical probe, than is minimally required to achieve diffusion control \(^{[46]}\). The response from films produced with more than 2 µL of the mixture appears to decrease and the sensitivity appears to
Figure 5.5:

Influence of layer 2 mixture volume on the sensitivity of the cholesterol response obtained with bi-layer PPy-NO₃/BSA-GLA-COD biosensor. Conditions were as described for Figure 5.4 except the layer 2 mixture volume was varied.
Table 5.3:

Influence of layer 2 mixture volume (BSA-GLA-COD) on the sensitivity of the cholesterol response obtained with bi-layer PPy-NO$_3$/BSA-GLA-COD biosensor

<table>
<thead>
<tr>
<th>Layer 2 Mixture Volume (L)</th>
<th>Drying Time (minutes)</th>
<th>Equilibration Time (minutes)</th>
<th>Sensitivity (mV/ M)</th>
<th>$R^2$</th>
<th>Linear Range [cholesterol] (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8</td>
<td>11</td>
<td>0.08</td>
<td>0.979</td>
<td>49.5 – 247.5</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
<td>24</td>
<td>0.099</td>
<td>0.994</td>
<td>49.5 – 247.5</td>
</tr>
<tr>
<td>4</td>
<td>24</td>
<td>42</td>
<td>0.07</td>
<td>0.991</td>
<td>49.5 – 247.5</td>
</tr>
<tr>
<td>6</td>
<td>38</td>
<td>60</td>
<td>0.07</td>
<td>0.992</td>
<td>49.5 – 247.5</td>
</tr>
<tr>
<td>8</td>
<td>50</td>
<td>87</td>
<td>0.068</td>
<td>0.992</td>
<td>99 – 247.5</td>
</tr>
<tr>
<td>10</td>
<td>60</td>
<td>116</td>
<td>0.05</td>
<td>0.988</td>
<td>99 – 247.5</td>
</tr>
</tbody>
</table>
plateau. This could be due to ineffective mass transfer that occurs with the thicker films, for reasons explained previously in Chapters 3 and 4. Interestingly, the achievable linear concentration range for cholesterol was influenced by variation of the layer-2 mixture volume. The films formed with a smaller volume of the layer 2 mixture were visibly thinner films and had the wider linear ranges of 49.5 – 247.5 μM. However, the thicker films formed by the use of larger volume mixtures for layer 2, were not linear at concentration below 99 μM, but achieved linear ranges of 99 – 247.5 μM.

Figure 5.6 illustrates the relationship between layer 2 mixture volume and drying time. Overall experiments using higher volumes of 6 – 10 μL for layer 2 films took longer to dry and were more time consuming as illustrated in Figure 5.6. The films had significantly longer equilibration times as illustrated in Figure 5.7. The equilibration time is measured as the time between placing the electrode into the buffer solution and obtaining a stable background potential. This is prior to the addition of the substrate. The increasing equilibration time with increasing film thickness at the higher volume for layer 2, may be due to the additional diffusion resistance [46].

Good diffusion properties are important for free movement of substrate and product and a good rate of reaction with the immobilised enzyme [234]. The highest sensitivities are achieved when there is large enzyme activity within a thin enzyme layer and an effective external mass transfer is provided [46].
Figure 5.6:

Influence of layer 2 mixture volume on the drying time of bi-layer PPy-NO₃/BSA-GLA-COD biosensor. Conditions were as described for Figure 5.4 except the layer 2 mixture volume was varied.
Figure 5.7:

Influence of layer 2 mixture volume on the equilibration time of the cholesterol response obtained with the bi-layer PPy-NO$_3$/BSA-GLA-COD biosensor. Conditions were as described for Figure 5.5.
Hence, the less sensitive responses observed could be caused by the increase in film thickness, which resulted from the use of higher mixture volume for layer 2. The thicker the film, the larger the diffusion barrier \cite{23} hence the amount of analyte reacting at the electrode surface is reduced which in turn reduces the sensitivity of the biosensor \cite{234,236-238}.

Another possibility for the decrease in sensitivity is deactivation or denaturing of the enzyme. This may be due to an increase in the toxic properties of GLA at higher GLA concentrations \cite{31,112}.

### 5.3.4 Interference Study

Figure 5.8 shows that the presence of ascorbic acid between 0 and 0.01 mM does not interfere significantly with the response of the bi-layer PPy-NO$_3$/BSA-GLA-COD biosensor. There was a 9.7% decrease in the sensitivity of the cholesterol response with the additions of 0.001 mM ascorbic acid. This decrease in sensitivity of the PPy-NO$_3$/BSA-GLA-COD is the lowest, in comparison with the results obtained for other biosensors in Chapters 3 and 4. The BSA-GLA-COD biosensor Chapter 3 had an enormous 70.3% decrease in sensitivity of the cholesterol response with the addition 0.001 mM ascorbic acid. The BSA-GLA-COD/BSA-GLA-CE biosensor in Chapter 4 had a 27.8% decrease in sensitivity towards cholesterol, at the same ascorbic acid concentration.
Figure 5.8:

Influence of ascorbic acid on the sensitivity of the cholesterol response obtained with the bi-layer PPy-NO₃/BSA-GLA-COD biosensor. Concentrations of components in sensing layer were as described in Table 5.1. Potentiometric measurement was made in 0.05 M phosphate buffer (pH 7.0).
Table 5.4:

Influence of [ascorbic acid] on the sensitivity of the cholesterol response obtained with the bi-layer PPy-NO$_3$/BSA-GLA-COD biosensor

<table>
<thead>
<tr>
<th>[Ascorbic Acid] (mM)</th>
<th>Sensitivity (mV/ M)</th>
<th>Percentage of Response Suppressed (%)</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.093</td>
<td>0</td>
<td>0.979</td>
</tr>
<tr>
<td>0.001</td>
<td>0.084</td>
<td>9.7</td>
<td>0.961</td>
</tr>
<tr>
<td>0.01</td>
<td>0.0601</td>
<td>34.4</td>
<td>0.986</td>
</tr>
<tr>
<td>0.1</td>
<td>0.0409</td>
<td>56</td>
<td>0.990</td>
</tr>
<tr>
<td>1</td>
<td>No Response</td>
<td>100</td>
<td>N/A</td>
</tr>
</tbody>
</table>
Figure 5.9 illustrates that the uric acid effect on the response of the biosensor followed the same trend as the different levels of ascorbic acid. At a concentration of 0.001 mM uric acid there was an 8.9% decrease in the sensitivity of the PPy-NO$_3$/BSA-GLA-COD biosensors to cholesterol. In Chapter 3 the BSA-GLA-COD biosensor which had a 52% decrease in sensitivity while the BSA-GLA-COD/BSA-GLA-CE biosensor in Chapter 4 which had a 33.9% decrease in sensitivity in the presence of the same concentration of uric acid. A possible explanation for these observations, is the presence of the inner PPy-NO$_3$ layer. The size exclusion properties of PPy coupled with the permselective properties of a bi-layer configuration seems to increase interferent rejection properties of the biosensor and reduced the effects of ascorbic and uric acids. Similar results were reported for glucose biosensors with the same bi-layer configuration of a PPy inner layer and a BSA-GLA-GOD outer-layer [291].

At ascorbic acid levels greater than 0.01 mM there appears to be a significant drop in the sensitivity of the biosensor, with a decrease between 34.4 – 56% due to suppression in the response. The presence of 1 mM ascorbic acid and 1 mM uric acid did not produce a measurable response because the response was completely suppressed. The background was very noisy and the potential did not stabilise. The suppression of the response at these concentrations occurred as a result of reasons mentioned previously.

The presence of ascorbic acid and uric acid did have an effect on the sensitivity of the PPy-NO$_3$/BSA-GLA-COD biosensor for cholesterol. However as mentioned in previous chapters the serum being analysed is
Figure 5.9:

Influence of uric acid on the sensitivity of the cholesterol response obtained with the bi-layer PPy-NO$_3$/BSA-GLA-COD biosensor. Conditions were as described for Figure 5.8.
Table 5.5:

Influence of [uric acid] on the sensitivity of the cholesterol response obtained with the bi-layer PPy-NO₃/BSA-GLA-COD biosensor

<table>
<thead>
<tr>
<th>[Uric Acid] (mM)</th>
<th>Sensitivity (mV/ M)</th>
<th>Percentage of Response Suppressed (%)</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.117</td>
<td>0</td>
<td>0.987</td>
</tr>
<tr>
<td>0.001</td>
<td>0.107</td>
<td>8.9</td>
<td>0.993</td>
</tr>
<tr>
<td>0.01</td>
<td>0.095</td>
<td>18.8</td>
<td>0.995</td>
</tr>
<tr>
<td>0.1</td>
<td>0.058</td>
<td>50.7</td>
<td>0.945</td>
</tr>
<tr>
<td>1</td>
<td>0.027</td>
<td>77.4</td>
<td>0.986</td>
</tr>
</tbody>
</table>
diluted in a 1:200 dilution. This high dilution was chosen to minimise the matrix effects of the serum as well as decrease the suppression effects of the ascorbic and uric acid [3]. The level of ascorbic acid in the diluted sample would be 0.23 – 0.45 μM [3] and the level of uric acid would be 0.7 – 2.1 μM [3] uric. It is therefore unlikely that there would be any suppression in the response by either interferent at these low concentrations. The most notable difference between this interference study and the previous studies in Chapters 3 and 4 is that the PPy-NO$_3$/BSA-GLA-COD biosensor appeared to have much better selectivity. This is indicated by the very small decreases in sensitivity in the presence of uric and ascorbic acids.

5.3.5 Linear Concentration Range and Minimum Detectable Amount

The bi-layer PPy-NO$_3$/BSA-GLA-COD biosensor is very sensitive with a minimum detectable amount of cholesterol of 2.5 μM. The first achievable linear concentration range for cholesterol with this biosensor, illustrated as “Linear Range 1” in Figure 5.10 (a) was between 2.5 and 25 μM cholesterol. The second linear range, illustrated as “Linear Range 2” in Figure 5.10 (a) appeared to be between 35 and 47 μM cholesterol. Comparison of the slopes of the two linear ranges, indicates that more sensitive quantification of cholesterol can be achieved at lower levels of cholesterol, within linear range 1. This is because the slope value for linear range 1 (0.9887) is approximately two and a half times as that of two linear range 2 (0.392). This result is similar to the BSA-GLA-COD and
Figure 5.10 (a):

Calibration curves obtained for the bi-layer PPy-NO$_3$/BSA-GLA-COD biosensor. The inlaid “Linear Range 1” graph shows that the sensor displays a linear response in the range of [2.5 – 25] µM cholesterol while the inlaid “Linear Range 2” graph shows that the sensor also displays a linear response in the range of [35 – 47] µM cholesterol.
the BSA-GLA-COD/BSA-GLA-CE biosensors in Chapters 3 and 4, respectively. An added advantage of the bi-layer PPy-NO₃/BSA-GLA-COD biosensor was that a third linear range was achievable, as illustrated in 5.10 (b) between 2.5 and 247.5 μM.

The reproducibility of fabrication of an electrode is equally as important as the reproducibility of measurements with an individual electrode. For this reason the sensitivity of the PPy-NO₃/BSA-GLA-COD biosensor over several different fabrications was analysed. The achieved sensitivity (0.1128 ± 14.9 %) mV/μM indicates that the PPy-NO₃/BSA-GLA-COD biosensor sensitivities given, were fairly consistent with repeated use of the biosensor over several different fabrications.

5.3.6 Percentage Recovery and Application to Serum Samples

Table 5.6 shows that excellent recovery, in the range of 92.1 - 100.7 % was achieved with the bi-layer PPy-NO₃/BSA-GLA-COD biosensor for the quantification of known spiked amounts of cholesterol. These results demonstrate that accurate and sensitive determination of cholesterol can be accomplished with the biosensor. The standard deviation values also demonstrate that the biosensor enables reproducible quantification.

Sensitive determination of cholesterol was achieved with the bi-layer PPy-NO₃/BSA-GLA-COD biosensor for the analysis of cholesterol in blood serum as shown in Figure 5.11. It also shows that the response to the addition of serum took longer to reach equilibrium than the standard, possibly due to some matrix effect. Table 5.7 shows the values obtained
Calibration Curves Obtained for the bi-layer PPy-NO$_3$/BSA-GLA-COD Biosensor. The graph shows that the sensor displays another linear response “Linear Range 3” in the range of [49.5 – 247.5] $\mu$M cholesterol.
Table 5.6:

Sensitivity of bi-layer PPy-NO$_3$/BSA-GLA-COD Biosensor in Percentage Recovery of Free Cholesterol

<table>
<thead>
<tr>
<th>[Cholesterol Added] (µM)</th>
<th>[Cholesterol Recovered] (µM)</th>
<th>Percentage Recovery (MD, n = 3) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.19</td>
<td>5.70</td>
<td>92.10 ± 2.63</td>
</tr>
<tr>
<td>12.38</td>
<td>12.38</td>
<td>100 ± 0.8</td>
</tr>
<tr>
<td>24.75</td>
<td>24.32</td>
<td>98.27 ± 0.58</td>
</tr>
<tr>
<td>49.50</td>
<td>49.84</td>
<td>100.70 ± 0.23</td>
</tr>
</tbody>
</table>
Figure 5.11:

Typical potentiometric responses obtained for the quantification of cholesterol in serum with the PPy-NO₃/BSA-GLA-COD biosensor. Cholesterol concentrations: (Std. 1) 6.2 μM; (Std. 2) 12.4 μM; (Std. 3) 18.6 and (Std. 4) 24.8 μM. Concentrations of components in sensing layer were as described in Table 5.1.
Table 5.7:

Determination of cholesterol in serum samples with a bi-layer PPy-NO₃/BSA-GLA-COD Biosensor

<table>
<thead>
<tr>
<th>Sample Number</th>
<th>[Cholesterol] measured by Clinical Method (mM)</th>
<th>[Cholesterol] measured by Biosensor (MD, n = 3) (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ser/P1 5006-4</td>
<td>4.09</td>
<td>3.77 ± 0.14</td>
</tr>
<tr>
<td>Ser/P1 5009-1</td>
<td>4.15</td>
<td>5.01 ± 1.78</td>
</tr>
<tr>
<td>Ser/P1 5006-2</td>
<td>4.04</td>
<td>3.56 ± 0.45</td>
</tr>
</tbody>
</table>
with this biosensor were not as close to the values obtained by the standard colorimetric method used at the Royal Prince Alfred Hospital. In comparison the values obtained with the BSA-GLA-COD/BSA-GLA-CE in Chapter 4 were closer to the values obtained by the standard colorimetric method.

As mentioned previously this method of analysis using biosensors has the advantage of being rapid, without the need for sample pre-treatment.

5.4 CONCLUSION

The optimum conditions for PPy layer 1 of the bi-layer PPy-NO\(_3\)/BSA-GLA-COD biosensor was a PPy-NO\(_3\) film polymerised at 0.25 mA/cm\(^2\) for 60 s. PPy layers polymerised for shorter periods of time were more sensitive than thicker PPy layers possibly due to better diffusion properties. A 2 µL volume of the mixture for layer 2 was chosen to give the optimum film thickness. With the addition of this volume an optimum drying time of \(~15\) minutes and an equilibration time of \(~24\) minutes were achieved. The optimum sensitivity of 0.099 mV/µM and \(R^2\) of 0.994 were also achieved at this film thickness.

The bi-layer configuration appeared to give sensitive responses. Achievable minimum detectable amount of cholesterol with the PPy-NO\(_3\)/BSA-GLA-COD biosensor is 2.5 µM which compares well with that obtained in Chapters 3 and 4. A linear concentration range of 2.5 – 25 µM was achieved in the most sensitive range. Recovery studies gave excellent results with percentage recoveries in the range 92.1 - 100.7.
This biosensor configuration was much more sensitive and reliable than the PPy-COD-[Fe(CN)$_6$]$^{4+}$ biosensor for cholesterol described in Chapter 2, with a much wider linear range and a much lower minimum detectable amount.

The PPy-NO$_3$/BSA-GLA-COD biosensor was successfully used for the determination of cholesterol in samples.
5.5 SUMMARY OF RESULTS

Table 5.8:

Results and optimum conditions obtained with the bi-layer PPy-NO₃/BSA-GLA-COD biosensor for free cholesterol measurement

<table>
<thead>
<tr>
<th>PARAMETER</th>
<th>OPTIMUM CONDITION / RESULT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Film Formation: <em>Inner PPy Layer 1</em></td>
<td></td>
</tr>
<tr>
<td>[Pyrrole]</td>
<td>0.1 M</td>
</tr>
<tr>
<td>[KNO₃]</td>
<td>0.1 M</td>
</tr>
<tr>
<td>Polymerisation Time</td>
<td>60 s</td>
</tr>
<tr>
<td>Applied Current Density</td>
<td>0.25 mA/cm²</td>
</tr>
<tr>
<td><em>Outer BSA-GLA-COD Layer 2</em></td>
<td></td>
</tr>
<tr>
<td>[Bovine Serum Albumin]</td>
<td>6.8% w/v</td>
</tr>
<tr>
<td>[Glutaraldehyde]</td>
<td>4.5% v/v</td>
</tr>
<tr>
<td>[Cholesterol Oxidase]</td>
<td>23 units/mL</td>
</tr>
<tr>
<td>Optimum Mixture Volume for Layer 2</td>
<td>2 L</td>
</tr>
<tr>
<td>Drying Time</td>
<td>~15 minutes</td>
</tr>
<tr>
<td>Equilibration Time</td>
<td>~24 minutes</td>
</tr>
<tr>
<td>Minimum Detectable Amount</td>
<td>2.5 M cholesterol</td>
</tr>
<tr>
<td>Linear Ranges [Cholesterol] ( M)</td>
<td></td>
</tr>
<tr>
<td>Linear Range 1</td>
<td>2.5 – 25 M</td>
</tr>
<tr>
<td>Linear Range 2</td>
<td>49.5 – 247.5 M</td>
</tr>
<tr>
<td>Percentage Recovery</td>
<td>92.1 – 100.7%</td>
</tr>
</tbody>
</table>
CHAPTER 6

CONCLUSIONS AND
RECOMMENDATIONS FOR
FUTURE WORK
CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK

6.1 CONCLUSIONS

The measurement of cholesterol was successfully accomplished by the fabrication of biosensors for cholesterol. This has been demonstrated by achieving excellent recoveries in percentage recovery studies and by accurate determination of cholesterol in serum samples. The methods that were developed during the course of this study were simple, efficient, sensitive, reliable and reproducible.

Initially a PPy-COD-[Fe(CN)₆]⁴⁻ biosensor was developed by incorporation of COD and a mediator, [K₄Fe(CN)₆], into a PPy film. The measurement of cholesterol was accomplished by amperometric and potentiometric detection. Potentiometric detection proved to be the more sensitive mode of detection with a minimum detectable amount of 12.4 μM and a linear concentration range of 2.5 - 247.5 μM. In comparison, amperometric detection had a minimum detectable amount of 49.5 μM and a linear concentration range of 49.5 - 198 μM. Hence, potentiometric detection was chosen as the optimum mode of detection for the measurement of cholesterol with all further biosensors that were fabricated. The use of chemical cross-linking with BSA-GLA as an alternative method of COD and/or CE enzyme immobilisation gave more sensitive responses.
to cholesterol than those obtained with the galvanostatic electropolymeryisation of COD in a PPy film.

A BSA-GLA-COD biosensor was successfully fabricated for the measurement of free cholesterol. The minimum detectable amount for this biosensor was \( \sim 2.5 \ \mu M \) which was almost five times lower than that obtained with the PPy-COD-[Fe(CN)₆]⁴⁺ biosensor. The achievable linear range was between 2.5 and 25 \( \mu M \). This means that more sensitive quantification of cholesterol can be achieved at very low concentrations with the BSA-GLA-COD biosensor. The biosensor was used successfully for percentage recovery studies with recoveries in the range of 94.3 – 110.5 and also for accurate determination of cholesterol in serum samples.

A bi-layer PPy based biosensor was constructed with a PPy-NO₃ as the inner layer and a BSA-GLA-COD cross-linked over it as the outer layer. This construction combines the positive permselective and interferent rejection properties of the PPy layer and the increased enzyme loading that can be achieved by the cross-linking method. The resulting PPy-NO₃/BSA-GLA-COD biosensor achieved a minimum detectable amount of \( \sim 2.5 \ \mu M \) cholesterol, which was almost five times more sensitive than that obtained for the PPy-COD-[Fe(CN)₆]⁴⁺ biosensor. The linear concentration ranges were 2.5 – 25 and 2.5 – 247.5 \( \mu M \) cholesterol. The presence of two linear ranges means that accurate determination of cholesterol can be achieved at the lower concentration range and that less sensitive
determination of cholesterol can be achieved at the higher cholesterol range.

These results for this sensor was similar to those obtained for the BSA-GLA-COD. However when the two sensors are compared in terms of the results obtained for the interference study, the response of the PPy-NO$_3$/BSA-GLA-COD seemed to suffer less suppression than the BSA-GLA-COD in the presence of the electroactive interferents. This suggests that the presence of the PPy layer help reduce interferences from substances, such as ascorbic and uric acid. The accuracy of the sensor was successfully demonstrated in percentage recovery studies where recoveries of 92.1 – 100.7% were achieved. The determination of cholesterol was also successfully accomplished in serum samples.

Total cholesterol measurement was also successfully achieved by co-immobilisation of CE and COD by cross-linking with BSA and GLA. A single layer configuration, where the COD and CE were immobilised in the one layer, was compared with a bi-layer configuration, where the CE was immobilised in the outer layer to enable the catalysis of the first hydrolysis step in the reaction. The bi-layer configuration was more sensitive than the single layer, possibly due to the CE being localised in the outer layer. The bi-layer BSA-GLA-COD/BSA-GLA-CE biosensor achieved a minimum detectable amount of 2.5 µM. The achievable linear ranges were 2.5 – 32.2 and 49.5 – 297 µM cholesterol. The presence of two linear ranges means that sensitive determination of cholesterol is possible at both low concentrations and less sensitive determination of cholesterol is also possible at higher concentrations.
Once again this biosensor gave an excellent accuracy with recoveries in the range 98.9 – 103.7 %. The biosensor was also successfully used for in the determination of cholesterol in serum samples.

6.2 RECOMMENDATIONS FOR FUTURE WORK

Future work could focus on the development of methods to minimise the effects of interferents, such as ascorbic and uric acid. Multilayer electrode configurations, using polypyrrole could also be investigated for improved interferent rejection and permselective properties. The modification and use of these biosensors for flow injection analysis could also be explored as a means of improving sample throughput for rapid analysis of a large number of samples.

The investigation of designs for the fabrication of a commercial prototype could also be considered. This may involve detailed study of the long-term stability of biosensors.


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   “www.heartfoundation.isa.net.au/docs/hhd2.htm


18. Heart Foundation of America Website:

"www.americanheart.org/cholesterol/about.jsp"


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